EVALUATION OF THE THERAPEUTIC POTENTIAL OF SELECTED HERBAL PLANTS FROM BARINGO COUNTY, KENYA, AGAINST *LEISHMANIA DONOVANI* IN BALB/C MICE

MARIA DIVINAH MOGAKA

I56/27320/2018

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE (APPLIED PARASITOLOGY) IN THE SCHOOL OF PURE AND APPLIED SCIENCES OF KENYATTA UNIVERSITY

SEPTEMBER 2023

DECLARATION

This thesis is my original work and has not been presented for the award of a degree or other award in any other University.

Signature ----------Date ----------

Maria Divinah Mogaka

Department of Zoological Sciences

Kenyatta University

SUPERVISORS

We confirm that the candidate performed the work presented in this thesis under our supervision.

Signature ……. Date……

Dr. Joshua Mutiso

Department of Zoological Sciences

Kenyatta University

Signature ……. Date…….

Prof. Michael Gicheru

Department of zoological sciences

Kenyatta University

DEDICATION

I dedicate this work to my family, especially my dad, John Mogaka, my son, Daniel, my sister, Gertrude, and friends. This work is also dedicated to all scientists for their unending search for solutions to human health problems, especially neglected diseases.

ACKNOWLEDGEMENTS

I thank God for guidance and strength throughout my academic and personal life. My gratitude goes to my father for always believing in me and encouraging me in my academic journey. I also appreciate my supervisors Prof. Gicheru and Dr. Mutiso for their immense support in proposal development, project execution, and thesis writing. This work was funded by the National Research Fund (NRF) through Professor Michael Gicheru. Special thanks to Mr. Macharia of IPR, Rebecca Ayako, Kenyatta University, and animal house attendant, Mr. Richard for their support in my laboratory work. I appreciate my friends Lucy, Nyorera, Omwenga, Winfred, and Susan for their encouragement and moral support.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS AND ACRONYMS

ANOVA	Analysis of variance
IPR	Institute of primate research
PBS	Phosphate buffered saline
WHO	World Health Organization
NMK	National Museums of Kenya
VL	Visceral leishmaniasis
MCL	Mucocutaneous leishmaniasis
CL	Cutaneous leishmaniasis
OECD	Organization for Economic Corporation and development
BCG	Bacillus Calmette Guerin
LD_{50}	Lethal dose
IC_{50}	Inhibition concentration
MEM	Minimum essential medium

ABSTRACT

Leishmaniasis is a parasitic infection caused by *Leishmania* parasites. Conventional chemotherapy remains the primary approach for treating leishmaniasis despite its associated drawbacks, such as high toxicity, frequent relapses, and the need for hospitalization. Conventional drugs are also expensive. Plant-based compounds offer promising alternatives to leishmaniasis treatment because they are effective, affordable, and less associated with toxicity and resistance. This research investigated the therapeutic potential of *Warburgia ugandensis*, *Prunus africana*, and *Piliostigma thonningii* against *Leishmania donovani* in vitro and in vivo. The anti-promastigote study was done by culturing promastigotes with the test compounds. For the toxicity study, Vero cells were incubated with different concentrations of the test compounds. Serum was obtained from the mice for total immunoglobulin gamma (IgG) quantification. In vivo studies were conducted by infecting the BALB/c mice with virulent *Leishmania donovani* promastigotes before commencement of treatment with methanolic extracts of *Warburgia ugandensis*, *Prunus africana*, and *Piliostigma thonningii* and control drug, sodium stibogluconate. Treatment with the test compounds significantly reduced the parasite burden. The outcome in the mice treated with plant extracts was comparable to those treated with pentostam ($P \geq 0.05$). In the promastigote assay, all the test compounds killed more than half of the promastigotes at the highest concentration (500µg/ml). *Warburgia ugandensis*, *P. thonningii*, and *P. africana* reduced the number of promastigotes from 2.0×10^6 to 7.7×10^3, 72.0×10^3, and 5.0×10^3, respectively. Sodium stibogluconate had the lowest IC50 (210 µg/ml), followed by *Warburgia ugandensis* (IC50 of 270µg/ml). *Piliostigma thonningii* and *P. africana* exhibited lower toxicity, with IC50 values of 720 µg/ml and 500 µg/ml, respectively. Treatment with the plant extracts resulted in low production of IgG antibodies, while the untreated control group showed higher IgG levels. Antileishmanial IgG antibody levels did not differ significantly with body weight changes. However, there was a strong significant correlation between levels of IgG antibodies and parasite numbers, indicating a possibility of the potential use of IgG levels in determining disease severity. Based on these results, the study concludes that *Warburgia ugandensis*, *Prunus africana*, and *Piliostigma thonningii* are safe and have antileishmanial activity against *L. donovani*. The study recommends the extrapolation of the present results in non-human primate disease model before clinical use of the compounds.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Leishmaniasis is a protozoan infection caused by *Leishmania* parasites, which are transmitted to humans by female *Phlebotomus* sandflies. More than 90 sand-fly species are proven or probable vectors worldwide (WHO, 2022). The parasite hosts are majorly vertebrates such as hyraxes, canids, rodents, and humans (Badirzadeh *et al*., 2020). Leishmaniasis occurs in Africa, Asia, Europe, and South and Central America. In Kenya, leishmaniasis is endemic in Baringo, Mwingi, Machakos, West Pokot, and Elgeyo Marakwet (Ouma and Mulambalah, 2021). Leishmaniasis accounts for about 1 million cases of parasitic diseases reported every year (WHO, 2022). The disease presents itself in three forms: Cutaneous (localized and diffuse), Mucocutaneous leishmaniasis, and Visceral leishmaniasis (Bezerra *et al*., 2018). Cutaneous leishmaniasis is common and is caused by *L. tropica*, *L. major*, and *L. braziliensis* species, and it presents in the form of skin ulcers. Cutaneous leishmaniasis cases every year are between 700,000 to 1 million (WHO, 2022).

Mucocutaneous leishmaniasis is characterized by ulcers that mutilate the mucosal membrane of the mouth and nose (Burza *et al*., 2018). Visceral leishmaniasis is characterized by fever, weight loss, anemia, and enlargement of the liver and spleen (Mann *et al*., 2021). Visceral leishmaniasis (VL) is fatal if left untreated; Up to 90 000 new cases of Visceral leishmaniasis are reported yearly (WHO, 2022). The current

treatment approach is primarily based on high doses of pentavalent antimonial compounds, which have been associated with various drawbacks and side effects such as nausea, liver toxicity, and kidney damage (Gidey, 2020). Treatment is done over a long period of time (28 days), which ends up being costly to the patients (Glans *et al*., 2018). Furthermore, antimonial compounds are toxic in nature, and there is a tendency for the disease to relapse even after successful chemotherapy regime with these compounds (Selvapandiyan *et al*., 2019). There is a need to develop other treatment options and especially from natural products. Medicinal plants are affordable, safe, and less associated with drug resistance (Jumba *et al*., 2015). They are used by most communities worldwide as the most economical and effective treatment options for parasitic diseases (Badirzadeh *et al*., 2020). Moreover, various studies have listed medicinal plants as potential treatment alternatives for leishmaniasis. *Warburgia ugandensis*, also known as the East African green heart, is commonly used in the African tropics and subtropics for treatment of malaria, cough, fever, and constipation, and among the Tugen tribe in Kenya, the bark is traditionally used to treat visceral leishmaniasis (Karani *et al*., 2013).

Previous studies have shown good antileishmanial activities of *W. ugandensis* against *Leishmania major* (Githinji *et al*., 2009; Ngure *et al*., 2014). *Piliostigma thonningii* has diverse chemical compounds, including flavonoids, tannins, and kaurene. Other compounds in *P. thonningii* are alkaloids, saponins, and volatile oils, which are responsible for its medicinal activities (Alfoyan *et al*., 2018). *Prunus africana*, which is also known as the African cherry is traditionally used to treat asthma and chest

complications (Karani *et al*., 2013). However, there is limited information on the laboratory efficacy of *W. ugandensis, P. thonningii,* and *P. africana* against *Leishmania dovonavi* parasites. The present study aimed to evaluate the therapeutic potential of *W. ugandensis, P. africana*, and *P. thonningii* against *L. donovani*.

1.2 Statement of the problem

Leishmaniasis is a common health problem, especially in the tropical regions of developing countries (WHO, 2022). The control of leishmaniasis involves vector control, vaccination, and chemotherapy. However, the current vector control measures have not been effective and till today, no vaccine against *Leishmania* has been developed successfully (Brito *et al*., 2017). In addition, current drugs for treating leishmaniasis have been shown to have high toxicity and relapse rates (Selvapandiyan *et al*., 2019). These drugs are not only toxic but are also very expensive (Gidey, 2020). Furthermore, these drugs must be administered over a long time in a hospital setting, therefore, they are not accessible to most patients who cannot afford to go to hospitals (Glans *et al*., 2018). Due to these setbacks associated with chemotherapy, the use of natural products is a better option because they are safe, economical, and easily accessible to the patients. *Warburgia ugandensis*, *P. africana*, and *P. thonningii* may offer better treatment options against leishmaniasis. However, no scientific studies have been done to show the merits of most of these plants in treating leishmaniasis.

1.3 Justification for the study

Despite being fatal and endemic, leishmaniases is a neglected disease and hence, there is the need for research on its control. Since vector control approach and effective vaccination is not available, there is need for development of new and effective control measures. Furthermore, the high toxicity rates and unavailability of pentavalent antimonials give more reason for research of much safer and available remedies for leishmaniasis. Natural plant products are cheaper, easier to access and therefore, are readily available in the communities even for people who cannot afford to go to the hospital. It is for these reasons that this study sought to evaluate the therapeutic potential of *W. ugandensis*, *P. africana*, and *P. thonningii* extracts from Baringo County, against *L. donovani* infection in the mouse model. This breed of mice is highly susceptible to visceral leishmaniasis and hence a relevant animal model for the proposed study.

1.4 Research questions

i. What is the effect of methanolic extracts of the bark of *W. ugandensis*, *P. africana*, and *P. thonningii* on *L. donovani* promastigotes in culture?
ii. What is the effect of methanolic extracts of the bark of selected herbal plants on visceral leishmaniasis in BALB/c mice?
iii. What is the effect of the methanolic extracts of the bark of selected herbal plants on the weight of *L. donovani* infected mice?

iv. What is the level of IgG production in *L. donovani* infected mice following treatment with the barks of the selected plants?

1.5 Hypothesis

i. Methanolic extracts of the bark of *W. ugandensis*, *P. africana*, and *P. thonningii*, selected from Baringo County have no effect on *L. donovani* promastigotes in culture.

ii. Methanolic extracts of the bark of the selected herbal plants from Baringo County have no effect on visceral leishmaniasis in BALB/c mice.

iii. Methanolic extracts of the bark of the selected herbal plants from Baringo County have no effect on the weight of *L. donovani* infected mice.

iv. Methanolic extracts of the bark of the selected herbal plants from Baringo County have no effect on IgG production in *Leishmania donovani* infected mice.

1.6 Objectives

1.6.1 General objective

To evaluate the therapeutic potential of *W. ugandensis*, *P. africana*, and *P. thonningii,* selected from Baringo County against *L. donovani* promastigotes in culture and visceral leishmaniasis in BALB/c mice.

1.6.2 Specific objectives

i. To evaluate the effect of methanolic extracts of the barks of *W. ugandensis*, *P. africana*, and *P. thonningii*, selected from Baringo County on *L. donovani* promastigotes in culture.

ii. To determine the effect of methanolic extracts of the bark of the selected plants from Baringo County on parasite load in visceral leishmaniasis BALB/c mice models.

iii. To determine the effect of methanolic extracts of the bark of the selected plants from Baringo County on the weight of *L. donovani* infected mice.

iv. To quantify IgG production in *L. donovani* infected mice following treatment with the barks of the selected plants from Baringo County.

1.7 Significance of the study

The present study aimed at obtaining an effective, less toxic, and readily available drug for leishmaniasis. The success of this study provides a solution for the urgent measures being sought to stop the numerous deaths that occur annually due to leishmaniasis. This study provides scientific validity of the herbal plants used by the local communities. Therefore, the traditional healers can provide scientifically proven medicine to their patients without a doubt that the herbs are effective. Moreover, it will draw the attention of the state or national government, non-governmental organizations, and research funding organizations to medicinal plant-based approach in the control of leishmaniasis and other related infections.

CHAPTER TWO

LITERATURE REVIEW

2.1 Leishmaniasis

Leishmaniasis is a vector-borne parasitic diseases caused by the *Leishmania* protozoa (WHO, 2022). There are over 90 vector species of the genus *Phlebotomus* and *Lutzomyia* that spread *Leishmania* parasites in the old and new world respectively (Colares *et al*., 2009). The disease occurs in Africa, Asia, Europe, South, and Central America. It is estimated to be endemic in 98 countries, and it is among the deadliest diseases after Malaria (Badirzadeh *et al*., 2020). New annually reported incidences of leishmaniasis are between 700,000 and 1 million cases, and up to 30,000 deaths (WHO, 2022). However, leishmaniasis is considered a neglected disease despite the fatalities it can cause.

Leishmaniasis presents in three clinical forms: cutaneous, visceral (Kal-Azar), and mucocutaneous leishmaniasis. The cutaneous form is characterized by an oriental sore, with lesions that may develop from papules or nodules (Cecílio *et al*., 2022). The lesions may develop to be ulcerative with a central depression and an indurated border, which later develops into an atrophic scar (Kayani *et al*., 2021). Mucocutaneous leishmaniasis is the disease form that is associated with destruction of the oropharynx and mucosa, resulting in extensive deformation of the mid face (Bezerra *et al*., 2018). It often causes obstruction and perforation of the cartilage around the mouth, nose, and the pharynx. The Visceral form is the most fatal and can lead to death if not treated timely.

2.2 Life cycle of leishmaniasis

Leishmaniasis is transmitted from human to human by infected female sand flies. Infection is initiated when an infected female sand fly is taking a blood meal, which is necessary for egg production. During a blood meal, the infected sand fly injects promastigotes into the host skin, which are ten phagocytized by macrophages present in the puncture wound, where they are transformed into the tissue form, amastigotes (Alemayehu, 2017). The tissue amastigotes multiply and invade other cells in the surrounding tissues. However, this depends on the parasite species involved. The development of symptoms and pathology depends on host and parasite factors; some people may remain asymptomatic. Tissue infection preference by the various parasites can vary but visceral leishmaniasis is usually associated with infection of the spleen, bone marrow, and liver macrophages, causing splenomegaly and hepatomegaly (Mann *et al*., 2021).

Sandflies are infected when they pick macrophages containing amastigotes from an infected host when obtaining a blood meal (Figure 2.1). The amastigotes change into the promastigote form upon reaching the mid gut, where they attach themselves with the aid of the lipophospoglycan layer that covers the parasite cell surface (Kayani *et al*., 2021). The promastigotes divide and move to the proboscis, where they wait to be inoculated into a potential host with the sand fly saliva. The sand fly saliva is known to enhance infectivity of some *Leishmania* parasite species and severity of the cutaneous lesions (Cecílio *et al*., 2022).

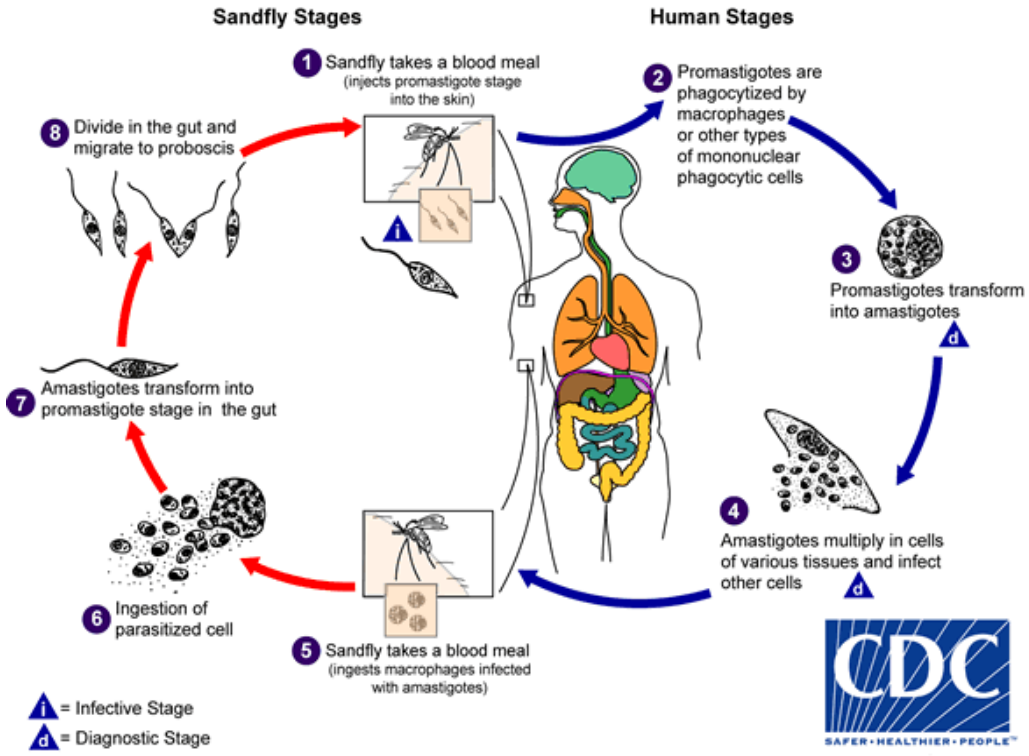

Figure 2.1: Leishmania parasite life cycle (CDC, 2020).

2.3 Clinical presentations

2.3.1 Cutaneous leishmaniasis

Cutaneous leishmaniasis is less life-threatening compared to other leishmaniasis forms. The etiology for cutaneous leishmaniasis majorly includes *L. tropica* and *L. major*. Other *Leishmania* species causing cutaneous leishmaniasis are *L. aethiopica* and *L. Mexicana* (Burza *et al*., 2018). The disease is characterized by an oriental sore, with

lesions that may develop from papules or nodules (Kayani *et al*., 2021). The lesions may develop to be ulcerative with a central depression and an indurated border, which later develops into an atrophic scar. The ulcers mostly occur on the face, extremities and other exposed body surfaces accessed and bitten by the sand fly.

The cutaneous form of leishmaniasis has extremely diverse clinical presentations which are determined by the parasite species and other host factors. The diverse clinical presentations of the cutaneous form are further categorized into localized and diffuse cutaneous leishmaniasis depending on how the ulcers and sores are spread on the host body (Torres *et al*., 2017). Diffuse cutaneous leishmaniasis is usually a non-ulcerative form that is very rare (Monge-Maillo and Lopez-Velez, 2015). The primary lesion develops as a papule, which in most cases is painless (Figure 2.2). Parasites migrate to different parts of the skin which results in disseminated nodules, especially on the face and extremities.

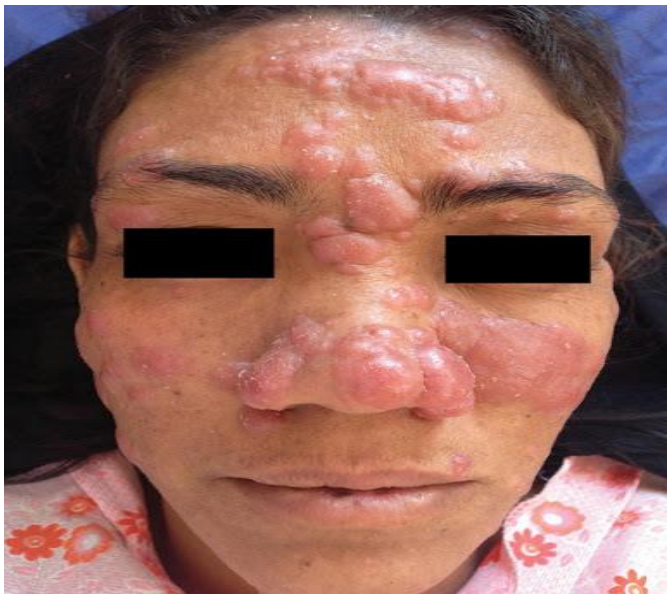

Figure 2.2: Diffuse cutaneous leishmaniasis. The image shows non-ulcerative nodules disseminated all over the face (Mehrolhasani, 2014).

Localized cutaneous leishmaniasis is not widely spread, the parasites are locally disseminated (Figure 2.3). A painless and heatless oriental sore form at the point of sand-fly bite, which later increases in size and develops into an open ulcer (Torres *et al*., 2017). The ulcer remains localized and may be wet or dry depending on the parasite species. For instance, sores by *Leishmania tropica* are often called dry-urban oriental sore because the sores are dry and mostly painless. Sores by *Leishmania major* are wet and are therefore commonly referred to as wet oriental sores (Marango *et al*., 2017). The early nodules resemble a boil and rapidly develop into a large ulcer, leaving a scar upon healing (Torres *et al*., 2017). However, some lesions will not develop into an ulcer, but rather persist as nodules or plague. Ulcers and scars due to cutaneous leishmaniasis, especially those caused by the old world, are usually self-healing within a

short time but may leave disfiguring scars, prompting the patient to suffer from permanent stigmatization (Glans *et al*., 2018).

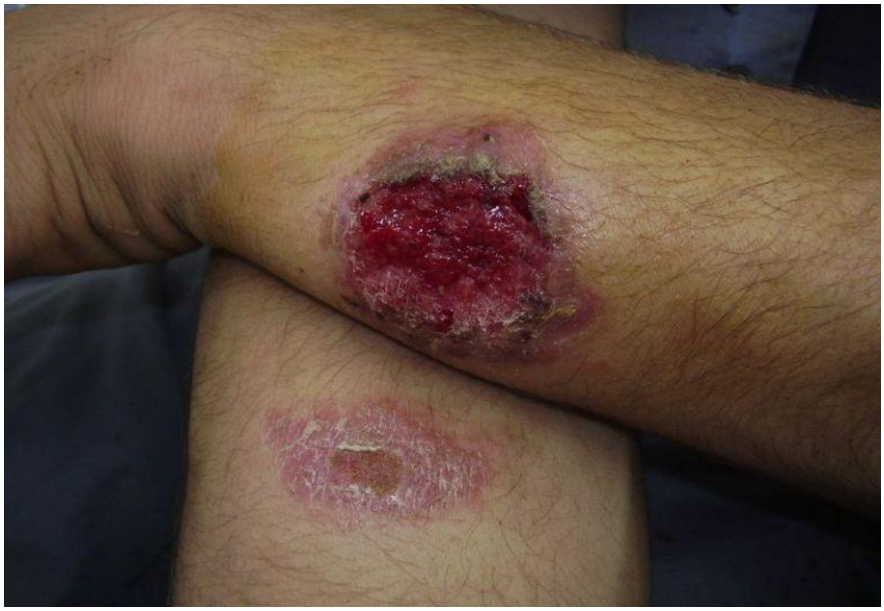

Figure 2.3: Localized cutaneous leishmaniasis. The image presents an enlarged cutaneous ulcer on the hands of a patient. The hand below presents a healing ulcer in the early stages of forming a scar (Pace *et al*., 2011).

2.3.2 Mucocutaneous leishmaniasis

The Mucocutaneous form of leishmaniasis causes the destruction of the oropharynx and mucosa, resulting in extensive deformation of the mid face. It often causes obstruction and perforation of the cartilage around the mouth, nose, and the pharynx (Mann *et al*., 2021). The lesions multiply and become large, leading to complete mutilation of the face (Figure 2.4). The major etiologic agent for this form of leishmaniasis is *Leishmania braziliensis* (Burza *et al*., 2018). The parasite causes lesions which appear months or

years from the time of exposure, which cause extensive tissue damage. The extensive tissue damage may pre-dispose the host to secondary diseases and other complications such as pneumonia (Abadías-Granado *et al*., 2021). In addition, the damage done to tissue may be permanent which exposes the host to stigmatization and may lead to depression.

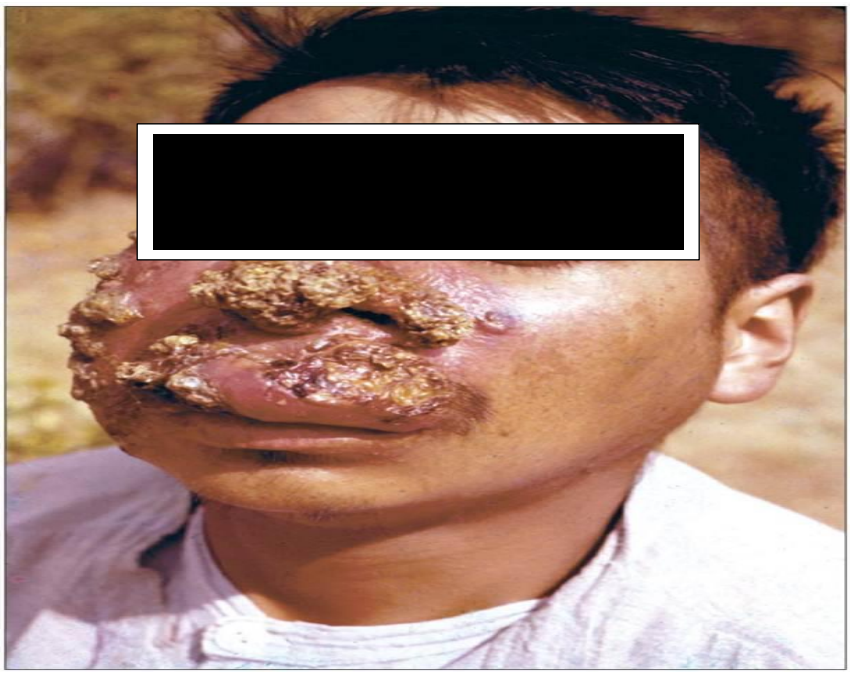

Figure 2.4: Mucocutaneous leishmaniasis. Image presenting a patient with seriously destroyed oropharynx and mucosa, which has resulted in extensive deformation of the mid face (Abadías-Granado *et al.*, 2021).

2.3.3 Visceral leishmaniasis

Visceral leishmaniasis is also called kal-azar and is the most fatal, chronic, and severe form among all the leishmaniasis forms (Gidey, 2020). According to WHO (2022), most incidences of VL are reported in some parts of South-east Asia, Brazil, and parts of East Africa. Countries affected are Kenya, India, Bangladesh, Somalia, Burma, and Sudan (Karani *et al*., 2013). There are 50 000 to 90 000 new cases of visceral leishmaniasis reported annually (WHO, 2022). The etiology of Kal-azar includes *Leishmania donovani* and *L. infantum* in the old world and *L. chagasi* in the new world, respectively (Savoia, 2015). This disease form is comprised of a broad range of clinical signs and may be fatal if the patient is left untreated (Sunyoto *et al*., 2018).

The most common signs of visceral leishmaniasis are irregular fevers, splenomegaly, hepatomegaly, weight loss, and anemia (Gidey, 2020). Abdominal swelling of the liver and spleen is the most prominent clinical sign of visceral leishmaniasis (Figure 2.5); swelling occurs because the parasites can invade the macrophages in these organs. Treatment of visceral leishmaniasis is successful but may relapse months or years after a successful regimen leading to formation of disseminated hypopigmented lesions throughout the body (Sundar and Agarwal, 2018). Leishmaniasis form that is associated with post-treatment relapse is called post kala-azar dermal leishmaniasis. In this case, the lesions do not develop into ulcerations, and they resemble nodules of lepromatous leprosy (Thakur *et al*., 2020).

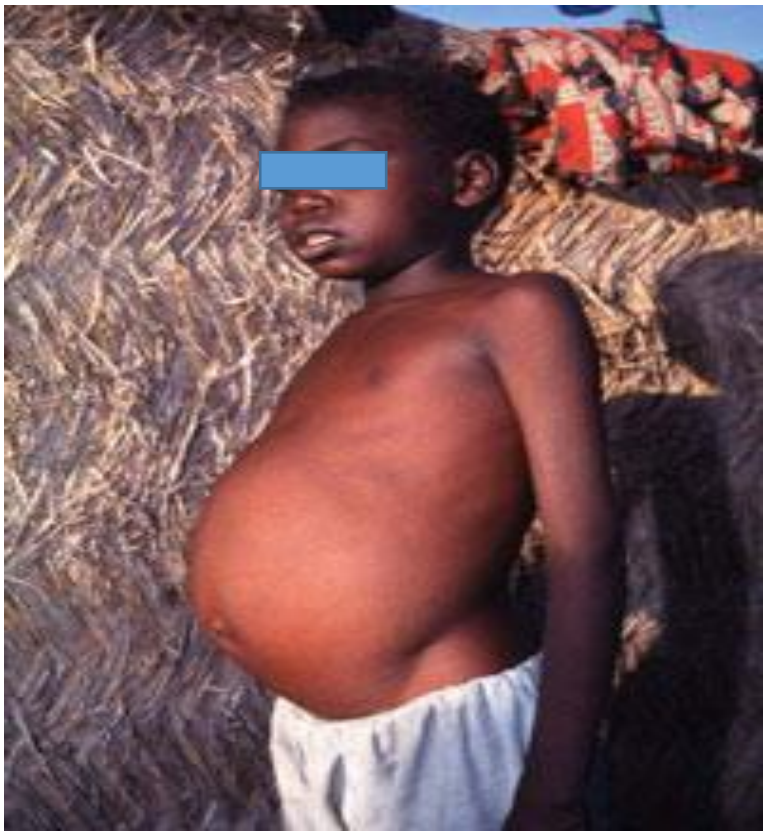

Figure 2.5: A child with hepato-splenomegaly caused by visceral leishmaniasis (Kishore, 2017).

2.4 Global burden of visceral leishmaniasis

Leishmaniasis occurs in five continents namely, Africa, Asia, Europe, South and Central America. The disease is predominant in poverty-stricken regions in Africa, Asia, and Latin America (Mann *et al.*, 2021). In these regions, leishmaniasis is associated with malnutrition, poor housing plans, deforestation, low immunity and most importantly, lack of resources (Torres *et al*., 2017). It is estimated to be endemic in 98 countries, and it is among the deadliest diseases after Malaria (Thakur *et al*., 2020).

There are 700,000 to 1 million cases of leishmaniasis reported annually, with 20,000 - 30,000 deaths reported every year (WHO, 2022).

Visceral leishmaniasis is the most fatal form and it exhibits significant distinctions in terms of the species involved from one region to the other. For instance, *L. donovani sensu sticto* is prevalent in the Indian region while *Leishmania donovani sensu lato* is found in the East African region (Gidey, 2020). The most common *Leishmania* species in Mediterranean, Asia, Middle East, and the rest of Africa is *L. infantum* while *Leishmania chagasi* occurs majorly in America. In America, visceral leishmaniasis is estimated to occur in 12 countries while more than 96% of the cases occurs in Brazil. Brazil reports between 4,200 and 6,300 cases of visceral leishmaniasis per year (Bezzear *et al*., 2016).

2.5 Leishmaniasis in Kenya

Kenya is known to be endemic for cutaneous and visceral leishmaniasis and cases of post kal-azar dermal leishmaniasis have been reported as well (Githinji *et al*., 2010). Visceral and cutaneous leishmaniasis are the commonest and although they are curable, the disease may cause high rates of morbidity and at times death. This is because of its low index of suspicion by both the host and the clinicians, poor disease management and late diagnosis (Gitari *et al*., 2018); its early symptoms can be nonspecific, resembling other common skin conditions or infectious diseases. These diseases majorly occur in the very poor nomadic tribes. Cutaneous leishmaniasis due to *Leishmania aethiopica* occurs majorly in the mountainous regions like Mt. Elgon, the

Rift valley escarpments and the Aberdares. These regions have increased risk of the disease due to extended grazing and farming practices which see the nomads sleep outside in the caves where the vectors are. Sandflies are nocturnal meaning they can bite the host at night or early morning (Cecílio *et al*., 2022) Cutaneous leishmaniasis caused by *Leishmania major* is prevalent in the lowlands such as Baringo County where both the adult and children are affected (Gitari *et al.,* 2018). Visceral leishmaniasis occurs in the rift valley province in the following counties: Baringo, West Pokot and Turkana. Visceral leishmaniasis is also endemic in the Eastern and Northeastern regions with the disease occurring in parts of Kitui, Machakos, Marsabit, Garrisa, Mandera, Wajir and Meru counties.

2.6 Animal models of leishmaniasis

Animals like mice and hamsters owe much of their popularity as models for biomedical research and have been used for leishmaniasis studies over a long period (Mears *et al*., 2015). Hamsters and mice models are known to be susceptible to different *Leishmania* species. The commonly used mice model for leishmaniasis is BALB/c mice. BALB/c mice have the ability for chronic disease development (Osada *et al*., 2016). The Syrian Hamsters are used as a murine model for leishmaniasis because of their high susceptibility to leishmaniasis and majorly due to their ability to develop pathological features of human disease (Mazire *et al*., 2021). The Syrian hamster is considered as the best animal model to study visceral leishmaniasis, however, their use is still limited due to the scarcity of the necessary reagents.

2.7 Immunology of leishmaniasis

In humans, immunity against leishmaniasis is mostly mediated by T lymphocytes. The T cells are responsible for generating specific cell mediated immune response in most parasitic infections, including leishmaniasis. The two types of T lymphocytes, The and Th2 secrete different types of cell-mediated immunity (Kumar, 2021). The Th1 lymphocyte secretes IF-γ, which induces the production of nitric oxide in macrophages, leading to destruction of the parasite while Th2 is responsible for secretion of cytokines such as IL-4, IL-5, and IL-10 which increase host susceptibility to infection and disease progression (Rossi and Fassel, 2018). Interferon gamma (IFN-γ) also induces IgG2 antibodies production (De Lima *et al*., 2021). However, the role of antileishmanial antibodies is not well understood. In visceral leishmaniasis, high levels of IgG are associated with high parasitemia, and studies have attributed the poor Th1 responses in VL to dominant Th2 immune response (Schwarz *et al*., 2013).

2.8 Diagnosis of leishmaniasis

Clinical manifestations of all the leishmaniasis forms are non-specific and therefore, resemble other skin diseases and conditions. For instance, cutaneous leishmaniasis produces lesions that resemble carcinoma, granuloma, myiasis and erythema while visceral leishmaniasis overlaps with diseases like malaria, schistosomiasis, trypanosomiasis, and malnutrition (Srivastava *et al*., 2011). Therefore, it is important to do prompt and definitive diagnosis to avoid over-diagnosis of leishmaniasis and to enable initiating appropriate treatment and control strategies.

2.8.1 Clinical diagnosis

Leishmaniasis is diagnosed based on clinical presentations. The different forms of leishmaniasis present different and diverse clinical manifestations. Some patients may remain asymptomatic but continue to transmit the disease. Cutaneous leishmaniasis is common and presents painless skin ulcers (Kayani *et al*., 2021). Symptoms appear a few weeks after infection and may last for years depending on the parasite species. In some patients, symptoms may not appear at all, or may appear months or years later. Mucocutaneous leishmaniasis presents with disfiguring ulcers like sores in the mouth, nose, lips, and face (Ibrahim *et al*., 2023). Symptoms appear years after the skin lesions which eventually develop into ulcerations. Other clinical manifestations of mucocutaneous leishmaniasis include runny/stuffy nose, nose bleeds and difficulty in breathing (Glans *et al*., 2018).

Visceral leishmaniasis manifests with fatal symptoms that appear months after the bite from an infected sand fly. Common clinical manifestations include weight loss, hepatomegaly, splenomegaly, lymphadenopathy, bleeding, and general body weakness (Gidey, 2020). A typical patient will complain of a fever two weeks after visiting an endemic area. Other rare symptoms are oedema, joint pains, vomiting and abdominal pains. It is important to know if the visceral leishmaniasis case is a primary case or a case resulting from a relapse from a previous infection. This is because the approach for treating and controlling the two cases is different. Clinical diagnosis is confirmed by

doing further laboratory tests such as parasitological tests and serological tests where applicable.

2.8.2 Laboratory diagnosis of leishmaniasis

Laboratory diagnosis is the most reliable way of confirming a disease because most methods rely on demonstrating the parasites. In the laboratory, the parasites can be demonstrated microscopically, by doing culture examination, isolation in animal models or by carrying out serological and molecular tests such as enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR), respectively (Reimão *et al.,* 2020).

2.8.2.1 Serology and molecular methods

There are several serological tests that are designed to detect antibodies produced by the immune system in the fight against *Leishmania* parasites. These serological techniques include immune florescent antibody technique (IFAT), and ELISA (Srivastava et al., 2011). However, these techniques are not appropriate for use in the field and instead, other serological tests like rK39 and direct agglutination tests (DAT) are used. The rK39 and DAT tests are appropriate for use in the field and treatment can commence once leishmaniasis has been confirmed with these tests. Polymerase chain reaction kits have been established to amplify the parasite's DNA, which can then be read visually without the need for sophisticated equipment (Sundar and Singh, 2018). Primers are used to amplify the target sequence.

2.8.2.2 Microscopic examination

Microscopic examination is achieved by using various tissue aspirates or biopsies from the spleen, liver, bone marrow, cutaneous lesions, or lymph nodes. Amastigotes are visible in the smears made from the aspirates and biopsies obtained when viewed under the microscope. The most used method is splenic impression smears which reveal amastigotes when stained with Giemsa stain and viewed under the microscope (Thakur *et al*., 2020). Viewing of the smears under the microscope should be done by an expert to ensure the viewed structures are amastigotes and nothing else. An experienced lab technician or scientist should look for the characteristics that are typical to the *Leishmania* amastigotes such as the size and shape, which is usually round or oval, presence of internal organs such as kinetoplast and the nucleus (Reimão *et al*., 2020). The nucleus and the kinetoplast are easy to view with Giemsa staining because the cytoplasm takes a blue color while the kinetoplast and nucleus take purple-pink color.

Culture examination is a desirable method of diagnosing leishmaniasis because it minimizes chances of misdiagnosis (Srivastava *et al*., 2011). Besides being the most desirable parasite confirmatory method, it is also used to preserve the parasite for a long time in culture for future use. Non-motile amastigotes are obtained from an infected host and cultured in a media containing blood and incubated for 3 days. Various media such as RPMI 1640 are used and are supplemented with fetal bovine serum. Amastigotes in the media transform into motile promastigotes which can also be viewed under a microscope. Culture examination can be coupled with isolation of the parasite in animal models.

In most cases, isolation of the parasite in an animal model precedes culture examination. Once the promastigotes are cultured, they are inoculated into an animal model, mostly BALB/c mice or into a hamster footpad or nose. This method is also used as a means of cultivating the parasites. Biopsies are obtained and evaluated histopathologically (Reimão *et al*., 2020). However, histopathology examination of biopsies alone is not enough to conclude on a diagnosis without identification of amastigotes.

2.9 Prevention, control, and treatment of leishmaniasis

2.9.1 Vector control

Vector control involves controlling sandflies, which are the primary transmission agents for *Leishmania*. Controlling vectors disrupts the disease or pathogen transmission cycle. Vector control may include spraying with insecticides, sleeping under insecticide–treated bed nets, environmental management, and using personal protection equipment (Montenegro *et al*., 2021). Although residual spraying in the houses is a simple and cost-effective vector control method, it is associated with high resistance rates; this is especially common in areas with prolonged use of insecticides.

2.9.2 Reservoir control

Various strategies have been devised for controlling the reservoirs for leishmaniasis. For example, killing infected dogs has been shown to be effective and is one of the forefront approaches in the Brazilian ministry of health Visceral leishmaniasis control programme. Killing reservoirs reduces the mean infectious period (Romero, 2014).

Dogs may also be vaccinated against the disease. Treating infected dogs is also a better leishmaniasis prevention option because killing dogs may be tedious and may raise ethical issues (Kaiming *et al*., 2018). Another approach is the use of insecticide-releasing dog collars and applying topical insecticides to scare away the vectors. However, most of these recommended vector-control strategies are unavailable or their efficacy and applicability has not been proven scientifically.

2.9.3 Leishmania vaccines

Killed *Leishmania* antigens have been found to be safe for use in vaccination against the parasite in several experimental models. However, they have low efficacies and hence require an adjuvant for successful vaccination (Arenas and Juarez-Duran, 2020). The first human *Leishmania* vaccine was done by scientists from the Institute of Endemic Diseases, University of Khartoum, Sudan. The vaccine was made of autoclaved *Leishmania major* promastigotes. They used Bacillus Calmette-Guerin (BCG) as the adjuvant. The activity or efficacy of the mixture of BCG and *L. major* promastigotes and was compared with BCG alone (Malvolti *et al.*, 2021). However, no vaccine has been developed against any form of leishmaniasis so far.

2.9.4 Current drugs for leishmaniasis

Treatment of leishmaniasis aims to minimize mortalities and morbidities associated with the various leishmaniasis diseases. The primary treatment approaches are based on toxic antimony compounds and other second-line drugs that are used when antimony compounds lack efficacy. Leishmaniasis is treated with pentavalent antimonial

compounds such as sodium stibogluconate (Pentostam), amphotericin B, and pentamidine. Others are meglumine antimoniate, paromomycin, and miltefosine (Chakravarty and Sundar, 2019). These drugs are administered over a long duration of time and are therefore, associated with toxic side effects on the patients. Some of the side effects associated with the antimony compounds are anorexia, vomiting, dizziness, fever, and myalgia (Gamboa-Leon *et al*., 2014). They are also costly, with their supply not being continuous due to low production. Moreover, their tendency to relapse after initial successive regime chemotherapy has posed a great challenge to the alleviation of the disease (Chakravarty and Sundar, 2019). The treatment of visceral leishmaniasis has greatly evolved owing to the numerous resistance patterns that have emerged over the years and due to the improving drug delivery systems.

2.9.4.1 Pentavalent antimonials

Treatment of leishmaniasis with pentavalent antimonials goes back to many decades ago (An *et al*, 2019). Pentavalent antimonials are made in two formulations, methylglucamine antimoniate and sodium stibogluconate. Despite being in use for over 70 years, their mode of action remains poorly understood. However, they treat leishmaniasis by inhibiting the glycolytic and oxidative pathways of fatty acids in amastigotes (Goto, 2012). Sodium stibogluconate (SSG), the current drug of choice was introduced in the market as a cheap and most effective antileishmanial drug and has been in use for decades. The recommended standard dosage for this drug by the world health organization in most parts of the world is 20mg/kg body weight, administered for 28-30 days (Sunder *et al*., 2018).

Sodium stibogluconate has poor oral absorption, therefore, it is given through intravenous or intramuscular routes (Reguera et al., 2019). This marks one of the very many disadvantages of sodium stibogluconate despite being the approved drug for treatment and management of visceral leishmaniasis. The patients require lengthy hospitalization owing to the routes used to administer the drug. Furthermore, the patients having to be injected every day is quite tedious to the healthcare giver and painful to the patient (Gidey, 2020). In addition, SSG is associated with serious side effects such as cardiac arrhythmias, myalgia, arthralgia, ventricular premature heartbeat, nausea, and vomiting (Moore and Lockwood, 2010). These side effects cause major limitation of the drug use in expectant women, older people, and patients with cardiovascular, renal, and liver disease (Goto 2012). Antimonial treatment regimen is more toxic in HIV patients and in a significant proportion of these patients, the major side effect has been chemical pancreatitis (sunder *et al*., 2018). Despite the associated toxicities and setbacks, sodium stibogluconate remains the standard drug for leishmaniasis treatment.

2.9.4.2 Paromomycin

Paromomycin is an aminosidine and categorized in the class of aminocyclitol-aminoglycosides. This drug not only comprises antiprotozoal activity against *Leishmania, Entamoeba* and *Cryptosporidium,* but also contains antibacterial activity An *et al.,* 2019). The common side effects of paromomycin are ototoxicity, pain at the injection site and it is known to increase liver enzymes (Goto 2012). In addition,

paromomycin is supposedly known to affect the fluidity of the plasma membrane and disrupts mitochondrial and ribosomal membrane, which leads to respiration inhibition.

2.9.4.3 Amphotericin B

Amphotericin B, also known as ambisome, is a polyenic antibiotic that contains leishmanicidal activity. It is an excellent drug targeting both the amastigotes and promastigotes by targeting the ergosterol found in the surface of the parasite (Goto, 2012). Therefore, the drug acts by increasing the parasite membrane permeability and increased influx of ions into the parasite cell. Ambisome exists in four different formulations. They include: deoxycholate, liposomal amphotericin B, amphotericin colloidal dispersion, and amphotericin B lipid complex.

Deoxycholate amphotericin B exhibits excellent clinical response but is associated with very adverse side effects, which limits its use. It is given by intravenous infusion and the common side effects are nephrotoxicity, infusion reactions, myocarditis, and bone marrow dysfunction (Sunder *et al*., 2018). Due to the prolonged administration and the associated toxicities, liposomal amphotericin B is the only formulation approved by the federal drug association (FDA) because it is rapidly absorbed by the reticuloendothelial tissues (Ickenstein and Garidel, 2019). Rapid absorption minimizes the amount of free drug available which in turn leads to significant reduction in drug toxicity. Amphotericin B dosage varies across the globe, but it is mostly given at 3-5mg/kg body weight daily for 6-10 days by infusion. The maximum dose for the entire treatment

period should not exceed 30mg/kg body weight. Ambisome is the first-line drug for severely ill patients, children, pregnant women, and the elderly people.

2.9.4.4 Miltefosine

Miltefosine is an oral alkyl phospholipid that was initially developed as an anticancer agent but later found to contain antileishmanial activity (Begoña and Rogelio, 2015). It is the only recognized oral agent against leishmaniasis and has demonstrated good efficacy against VL in India, Nepal, and Bangladesh. However, numerous failures have been reported among individuals with visceral leishmaniasis and HIV co-infections. Miltefosine is available in 10mg and 50mg capsules and are administered at dosages of 50mg twice each day for 28 days for patients weighing above 25kgs. Children between 2 and 11 years old are given a dose of 2.5mg/kg body weight for 28 days (Sundar, 2018).

2.10 Natural products against leishmaniasis

Medicinal plants are affordable, safe, and less associated with drug resistance (Deresa *et al*., 2022). They are used by most communities worldwide as the most economical and effective treatment options for parasitic diseases (Badirzadeh *et al*., 2020). Moreover, various studies have listed medicinal plants as potential treatment alternatives for leishmaniasis. Most plants have essential oils and volatile compounds in their flowers, leaves, stems, barks, roots, woods, fruits, and seeds. Some of the plants with these compounds are used to treat leishmaniasis and other diseases in various parts of the world (Colares *et al*., 2013). Plants belonging to the family Solanaceae, such as *Saracha*

punctata have shown inhibitory activities against *Leishmaniasis species* (Manuel *et al.*, 2001). Ethanolic extracts of *Desmodium gangeticum* have chemoprophylactic and chemotherapeutic properties against visceral leishmaniasis (Mishra *et al.,* 2005). Extracts of *Echinacea purpurea* have also shown immunomodulatory activities; they stimulate certain immune functions such as phagocytic activity of macrophages, and have also shown leishmanicidal activity (Soudi *et al.,* 2007).

2.11 Selected herbal plants from Baringo County

Several plant materials are found locally in Baringo County. Residents around the villages of this County use these plant materials for the treatment of leishmaniasis, among other diseases. Some of them have been proven to be effective in treating leishmaniasis locally, without the patient going to the hospital. *Piliostigma thonningii* has alkaloids, saponins, and volatile oils, which are responsible for its medicinal activities (Alfoyan *et al*., 2018). *Prunus africana*, which is also known as the African cherry is traditionally used to treat asthma and chest complications (Karani *et al*., 2013). However, these plant materials, including three selected plants have not been scientifically tested and evaluated against visceral leishmaniasis. *Piliostigma thonningii*, *Warburgia ugandensis*, and *Prunus africana* have not been tested in the laboratory to establish their safety and efficacy levels as a potential applicability in the treatment of visceral leishmaniasis.

2.12 Future perspectives in treatment of leishmaniasis

Based on the above literature review, it is evident that there is limited research on natural products with potential for treatment of leishmaniasis. Additionally, the selected herbal plants have not been evaluated for their potential use in the treatment of visceral leishmaniasis. Given the limitations associated with the current chemotherapies against leishmaniasis and the high safety levels of natural products, future effective and safe treatment against leishmaniasis should be derived from plant-based compounds. Therefore, the present study provides guidelines on the use of natural products to treat visceral leishmaniasis.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study area

The study was conducted in the Parasitology and Immunology laboratory, at the Department of Zoological Sciences of Kenyatta University and at the Institute of Primate Research (IPR). The laboratories have well established infrastructure with basic equipment and facilities for biomedical research.

3.2 Collection and preparation of plant materials

Barks of *P. thonningii, W. ugandensis,* and *P. africana* were collected from various villages in Baringo County with the help of traditional healers and community members. Voucher specimens of the plant parts were taken to the East African Herbarium in Nairobi for identification and future reference. The barks of the plants were air-dried for 15 days in a shed and then ground into a powder using an electric mill. Methanolic extraction was done by soaking 500g of the powder in absolute methanol for 72 hours before filtering, followed by solvent recovery under vacuum in a rotary evaporator, at 30-35^{0}C. The extracts were packed into airtight storage bottles and stored at -20^{0}c.

3.3 Preparation of *Leishmania donovani* parasites for infection

The *Leishmania donovani* strain NLB-065 obtained from the spleen of an infected patient and maintained by intracardiac hamster-hamster passage at the Institute of primate research was used in this study. A hamster splenic aspirate was cultured in Schneider's Drosophila insect medium, supplemented with 20% fetal bovine serum and 100 µg/ml of Gentamicin at 25^0c. Harvesting of the *L. donovani* promastigotes was done during their stationary phase, followed by centrifugation at 2500 rpm for 15 minutes at 4^0C. The parasites were washed thrice with sterile PBS and counted before use for the study.

3.4 Experimental design

The study involved both in vitro and in vivo evaluation of the test compound**s**. For in vitro promastigote studies, 200 µl of Schneider's Drosophila media was added into each of the microtiter plate wells containing the highest concentration (500µg/ml) of the plant extracts and control drug. In the subsequent wells, only 100µl of the media was added (2 to 12). Two microliters of each test compound were put into the first wells and diluted serially in the subsequent wells; the concentrations ranged from 500 µg/ml to 3.9 µg/ml. One hundred microliters of culture medium containing 2.0 x 10^6 stationary-phase *Leishmania donovani* parasites were added to each test well. Tests were performed in triplicates for each test compound concentration. The untreated control group contained only media and promastigotes. The starting concentration of the test compounds was 500µg/ml. Parasite observation and counting were done using a

microscope. The results were expressed as parasite count following a 72-hour incubation period. Toxicity test was done by incubating Vero cells with different concentrations of the test compounds for 72 hours, before assessment of growth inhibition. The starting concentration of the test compounds was 1000 µg/ml. For in vivo experiment, inbred BALB/c mice, 6-8 weeks of age of both sexes were acquired from the Department of Zoological Sciences, Kenyatta University. The mice were kept in the rodent house and provided with food and water every day. Mice were grouped into six groups, each comprising of 6 mice of both sexes. The mice in each group were marked appropriately for group identification with each of the groups having separate cages.

Virulent promastigotes ($2x10^6$) were injected into the mice in group I, II, III, IV and V intraperitoneally. The mice were kept for 5 weeks for the disease to develop. Group I, II and III were treated with plant extract *P. thonningii, W. ugandensis,* and *P. africana,* respectively. Group IV mice were treated with the reference control drug, sodium stibogluconate. Treatment with the plant extracts and control drugs commenced on the 36^{th} day post infection and were done intraperitoneally at a dose of 20mg/kg body weight once daily for 18 days. Group V was infected, but untreated control. Group VI was included as a naïve control. Body weights of all mice were taken at day 0, 6, 12 and 18 during the treatment period. One day after the last treatment, the mice were sacrificed and blood samples were taken for serum preparation for measurement of

antileishmanial antibodies, while the spleen were obtained for preparation of splenic impression smears for parasite burden quantification by microcopy.

3.5 Measurement of body weights

All mice were weighed before commencement of treatment. Subsequent weights were taken on days 6, 12, and 18 during the treatment period. They were weighed using an electronic weighing balance. Body weight changes were determined by obtaining the difference of mice weights taken at day 0 and day 18 of treatment. The efficacy of the test compounds was evaluated by comparing body weights of the treated mice with mice in the control groups.

3.6 Plant extracts toxicity assay

The toxicity study was done by culturing Vero cells in complete minimum essential medium (MEM), as described (Githinji *et al*., 2010). The study was done in duplicates in a microtiter plate. The Vero cells were cultured at 37^0C in 5% CO_2 for 24 hours to attach to the microtiter wells. Afterwards, the medium was aspirated off before 100µl of the plant extracts and sodium stibogluconate was added and serially diluted across the wells. The starting concentration of the test compounds was 1000 µg/ml. Further incubation at 37^0C was done for 72 hours. The controls used were Vero cells and medium with no extract. Ten microliters of 3-(4,5-Dimethylimidazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent was added to each well and the cells were incubated for 3 hours until a purple precipitate was visible under a microscope. The

medium together with MTT was aspirated, after which 100µl of dimethyl sulfoxide (DMSO) was added and plates shaken for 5 minutes. Absorbance was measured for each well at 562nm using a microtiter plate reader. Cell viability (%) was calculated at each concentration using the following formula:

$$\mathrm{CV} = \frac{A_1 - B_1}{A_2}\ x\ 100.$$

Where, CV is Cell Viability (%), A_1 is the average absorbance in duplicate drug wells, A_2 is the average absorbance in control wells, and B_2 is the average blank wells (Cobb, 2019).

3.7 Anti-promastigote evaluation of test compounds

Leishmania donovani parasites were obtained at the stationary phase, counted, and suspended in culture medium at 2.0 x 10^6 parasites/ml. The tests were performed as described (Mutiso *et al*., 2011). The tests were performed in 96-well microtiter plates maintained at 26°C. Two hundred microliters of complete Schneider's Drosophila medium were put in the wells containing the maximum concentrations of the compounds and 100 µl in the next wells (2 to 12) and control; 2 µl of compound solutions of 20 mg/ml in distilled water was added to wells number 1 and diluted serially; the concentrations ranged from 500 µg/ml to 3.9 µg/ml. One hundred microliters of culture medium containing 2.0 x 10^6 stationary-phase *Leishmania donovani* parasites were added to each test well. The starting concentration of plant

extracts was 500µg/ml. Tests were performed in triplicates for each test compound concentration. The untreated control group contained only media and promastigotes. Parasite observation and counting were done using a microscope. The results were expressed as infection rates following a 72-hour incubation period.

3.8 Estimation of parasite burden in infected BALB/c mice following treatment

Leishmania donovani promastigotes were injected into inbred BALB/c mice intraperitoneally. They were left for 5 weeks for the disease to develop. The infected mice were then grouped into five groups and treated as follows: Group I, II and III were treated with plant extracts *P. thonningii, W. ugandensis*, and *P. africana*, respectively. Group IV mice were treated with the reference control drug, sodium stibogluconate. Treatment with the plant extracts and control drugs commenced on the 36th day post infection and were done intraperitoneally at a dose of 20mg/kg body weight once daily for 18 days. Group V was the negative control, which was infected but not treated. A day after the last treatment, the mice were sacrificed, and the spleen obtained for preparation of splenic impression smear for parasite burden quantification by microcopy. The amastigotes were counted against 1000 splenocytes and expressed as percentage parasite count.

3.9 Quantification of antileishmanial total IgG antibodies

Total IgG antibodies were quantified using enzyme linked immunosorbent assay (ELISA). Coating of polystyrene Micro-ELISA plates was done overnight with one

hundred microliters of soluble *L. donovani* antigen at 10 µg/ml in Carbonate-bicarbonate buffer (pH 9.6). This procedure was then followed by blocking the nonspecific binding sites in the plates with 3% bovine serum albumin (BSA) in phosphate buffered saline (PBS). The plates were incubated for one hour at 37^0c. They were then washed 4 times with the wash buffer before adding 100 µl of the mice serum. The plates were incubated further for 2 hours at 37°C. The plates were washed four times again followed by addition of diluted horse radish peroxidase-conjugated anti-mouse-IgG antibody (Amersham) and incubation for 1 hour at 37°C. Tetramethylebenzidine (TMB) microwell peroxidase substrate was added afterwards and the plates incubated for 20 minutes in the dark. The plates were then read at 630nm in a micro-plate and results presented as optical densities.

3.10 Statistical data analysis

The data collected from the in vivo and in vitro studies were analyzed using GraphPad prism 8, 2018 software. Data on weight changes between day 0 and 18 of treatment was analyzed using paired t-test. Anti-promastigote assay, IgG production test, and toxicity assays were conducted in triplicates, and the obtained data was analyzed with one-way (ANOVA) followed by Tukey post hoc test. In vivo evaluation of each plant extract against leishmaniasis was done in 6 mice and differences between amastigote numbers were analyzed by one-way ANOVA. The correlation between the groups treated with various plant extracts was determined by Spearman's rank correlation test. A p-value of < 0.05 was considered statistically significant.

CHAPTER FOUR

RESULTS

4.1 *In-vitro* bioassays

4.1.1 Anti-promastigote activity of the test compounds

The efficacy of the test compounds was determined by incubating the parasites together with the test compounds. The results on efficacy of the plant extracts against promastigotes are indicated in Figure 4.1. The parasite count after incubation with 500µg/ml of *P. africana* was 5.0×10^3, which shows a more than half decrease from the initial concentration (2.0×10^6). *Warburgia ugandensis* was the second most effective plant extract against promastigotes at the highest concentration, since it reduced the number of promastigotes from the initial concentration of 2.0×10^6 to 7.7×10^3.

Piliostigma thonningii was the least effective compared to all the test compounds because it killed the least number of promastigotes at the highest concentration, reducing the parasite number from 2.0×10^6 to 72.0×10^3. However, at the highest concentration, all the test compounds killed more than half of the promastigotes. At 500µg/ml, data analysis indicated a significant difference between the test compounds and the untreated control group, which had only the promastigotes in media ($p<0.0001$). Tukey's multiple comparison test showed a significant difference between *W. ugandensis* and *P. thonningii*, and between *P. africana* and *P. thonningii* ($P<0.05$).

However, there was no significant difference between *W. ugandensis* and *P. africana* (P=0.9976).

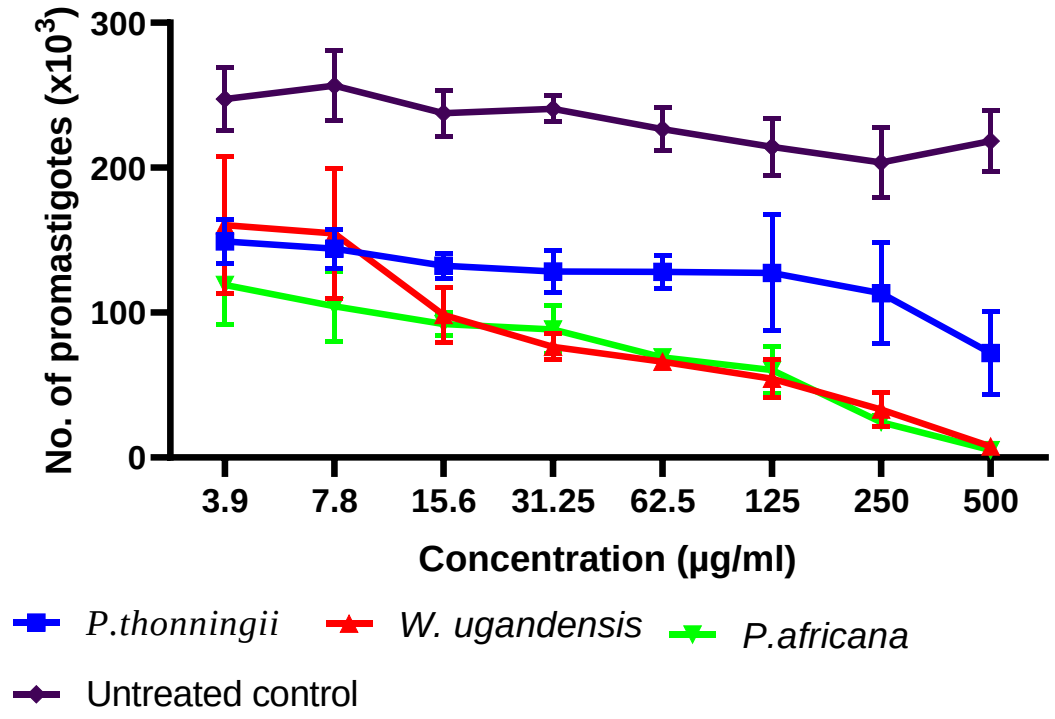

Figure 4.1: Number of promastigotes following treatment of *L. donovani* cultures with various test extracts. Promastigotes were incubated with varying concentration of the test drugs for 72 hours and then counted to determine the efficacy of the drugs against promastigotes. The data in the graph shows the mean number of promastigotes after 72 hours incubation with *P. thonningii*, *W. ugandensis*, and *Prunus africana*.

4.1.2 Cytotoxicity levels of test plant extracts and sodium stibogluconate

Drug cytotoxicity was measured by Vero cells death after incubating them with the test drugs for 72 hours. *Warburgia ugandensis* killed 50% of the Vero cells at a concentration of 270µg/ml, which was lower compared to the concentration of other plant extracts. *Piliostigma thonningii* and *Prunus africana* killed 50% of the cells at a

concentration of 720µg/ml and 500µg/ml, respectively. The standard drug, sodium stibogluconate was the most toxic drug with an inhibition concentration reducing parasite numbers by 50% (IC50) of 210 µg/ml (Table 4.1). There was a significant difference in cell viability between the wells treated with *P. thonningii* or *Prunus africana* when compared to sodium stibogluconate ($P<0.05$). The mean optical densities (ODs) for *Piliostigma thonningii* and *W. ugandensis* at 1000µg/ml were 0.2590 and 0.1765, respectively. The ODs for *Prunus africana*, sodium stibogluconate, and untreated control at 1000µg/ml were 0.1090, 0.1590, and 0.0680, respectively. The Cell viability in the wells treated with *W. ugandensis* was not significantly different from sodium stibogluconate ($P>0.05$). Tukey's multiple comparison test showed a significant difference between the toxicity levels of *Piliostigma thonningii* and *Prunus africana.* There was also a significant difference between *Piliostigma thonningii* and *Warburgia ugandensis,* and between *W. ugandensis and P. africana* ($P<0.05$).

Table 4.1: IC50 values of test plant extracts

Compound	IC50 (µg/ml)
P. thonningii	720 µg/ml
P. africana	500 µg/ml
W. ugandensis	270µg/ml
Sodium stibogluconate	210 µg/ml

4.1.3 Antileishmanial antibodies and their relationship with other parameters

4.1.3.1 Effect of the selected plant extracts on IgG levels in *L. donovani* infected mice

Treatment of infected mice with *W. ugandensis* induced the lowest production of IgG antibodies as indicated by low mean optical density (0.1268) while *Prunus africana* induced the highest level of IgG (OD = 0.1356) among the treatment groups as shown in Figure 4.2. There was high production of IgG antibodies in the untreated mice as indicated by a higher optical density of 0.2379. However, the amount of IgG produced among the treatment groups, including the standard drug, sodium stibogluconate did not differ significantly ($P>0.05$). The difference in the levels of IgG produced in mice treated with *Prunus africana* and *W. ugandensis*, and the untreated control was significant ($P=0.0002$).

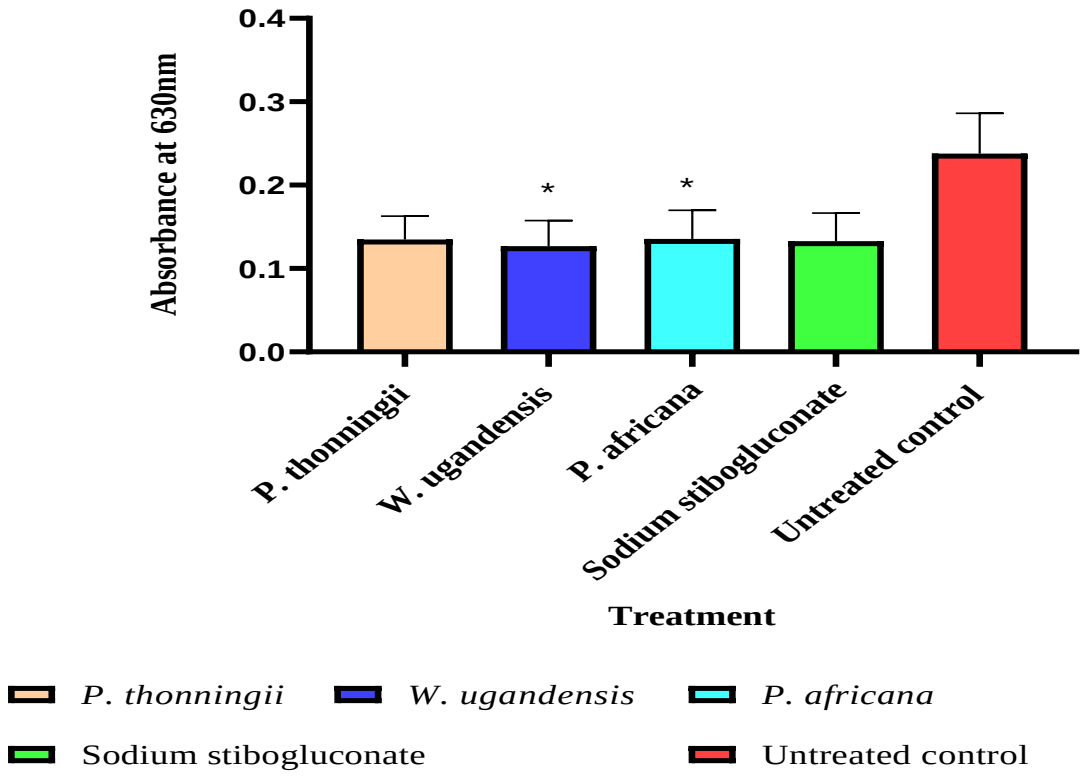

Figure 4.2: IgG production in *L. donovani* infected mice following treatment with *P. thonningii, W. ugandensis,* and *Prunus Africana*. Mice were infected and left for five weeks for the disease to develop. Afterwards, they were treated with *various* plant extracts and one day after the last treatment, blood samples were taken from all mice for serum preparation for measurement of antileishmanial antibodies**.** The graph represents mean IgG optical density (OD) values ± standard deviation.

4.1.3.2 Relationship between IgG levels and parasite burden

Following *L. donovani* infection and treatment of mice with various plant extracts and a control drug, the results showed a positive correlation between parasite load and the level of IgG produced. A higher percentage parasite count was associated with a higher absorbance (OD value) (Figure 4.3). The untreated group, with the highest mean OD (0.2379) levels had the highest percentage of parasite count (95.4%). Groups treated with *W. ugandensis* and sodium stibogluconate had the lowest percentage parasite count

and IgG production. There was a significant positive correlation between the mean percentage parasite count and IgG antibody production (r=0.9880, P=0.0016)

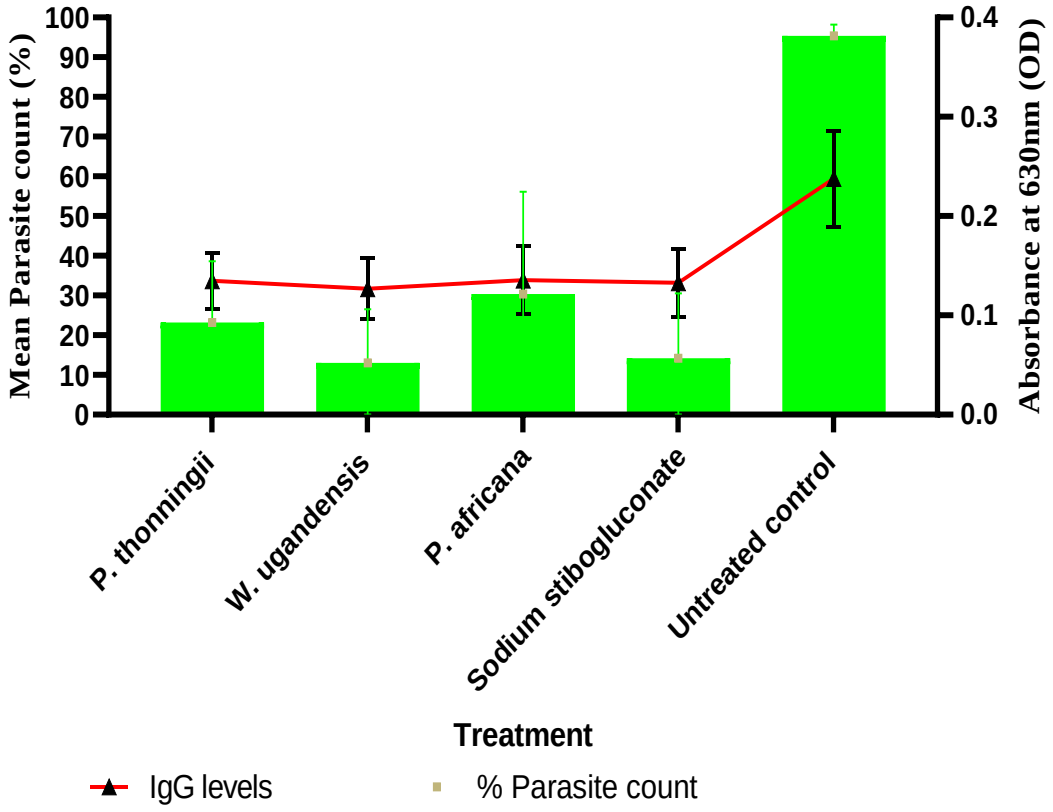

Figure 4.3: Relationship between mean percentage parasite count and *L. donovani* IgG levels. Mice were infected with *L. donovani* and left for five weeks for the disease to develop. They were treated with various plant extracts and a day after the last treatment, blood samples were taken from all mice for serum preparation for measurement of antileishmanial antibodies**.** Spleen was obtained for preparation of splenic impression smears for parasite quantification. The graph shows the relationship between parasite load and IgG production.

4.2 Weight changes and its relationship with IgG levels

4.2.1 Weight changes in mice following treatment

Mice weights taken at day 0 before treatment and 18 days after the initial treatment ranged from 32.58±4.62g, which was the lowest measurement taken from the group treated with *W. ugandensis* to 38.48±2.85g, which was the highest value taken from the untreated control (Figure 4.4). There was a notable decrease in weight in the group treated with sodium stibogluconate from day 0 to 12 (35.98g to 32.00g). Though all mice groups indicated a slight increase in weight between day 12 and 18, the weights taken on day zero before treatment and day 18 after the initial treatment day were not significantly different (P>0.05).

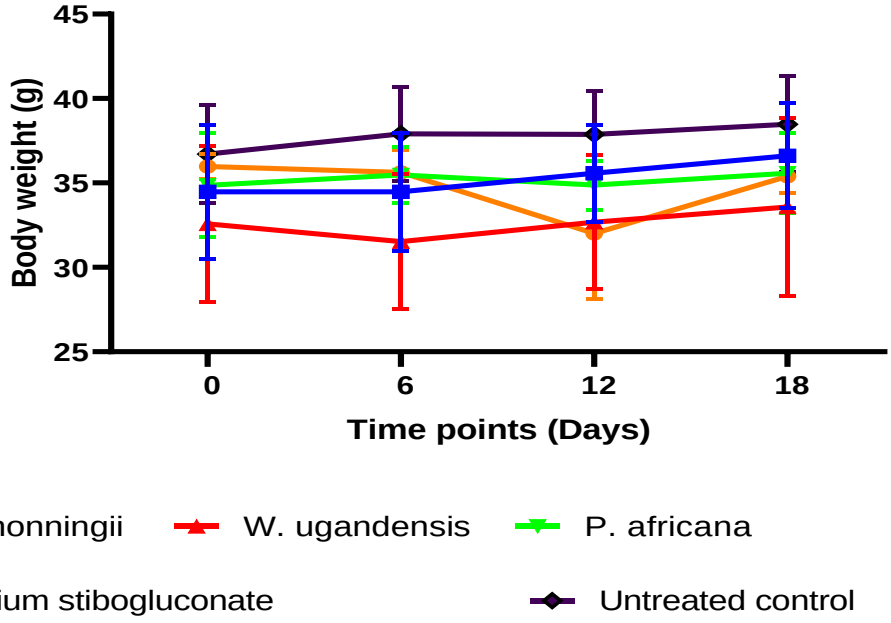

Figure 4.4: Mean weights of different treatment and control groups at day 0 to 18 of treatment. Weights of mice were taken before treatment and on day 0, 6, 12, and 18 during the treatment period. The data shows the mean weight of mice treated with various plant extracts and the controls on day 0, 6, 12, and 18 of treatment.

4.2.2 Relationship between IgG levels and body weights

Treatment groups with higher body weights on day 18 of treatment indicated higher levels of IgG. The results showed a positive correlation between the mean mice weight and IgG levels (Figure 4.5). The untreated control group, which had the highest mean body weights (38.48g). had high levels of IgG as indicated by a mean OD of 0.2379. Mice treated with *W. ugandensis* had the lowest mean body weight (33.58g) and lowest mean OD (0.1268). There was no significant correlation between the mean body weights of mice and IgG antibody production (r= 0.8334; p=0.0795).

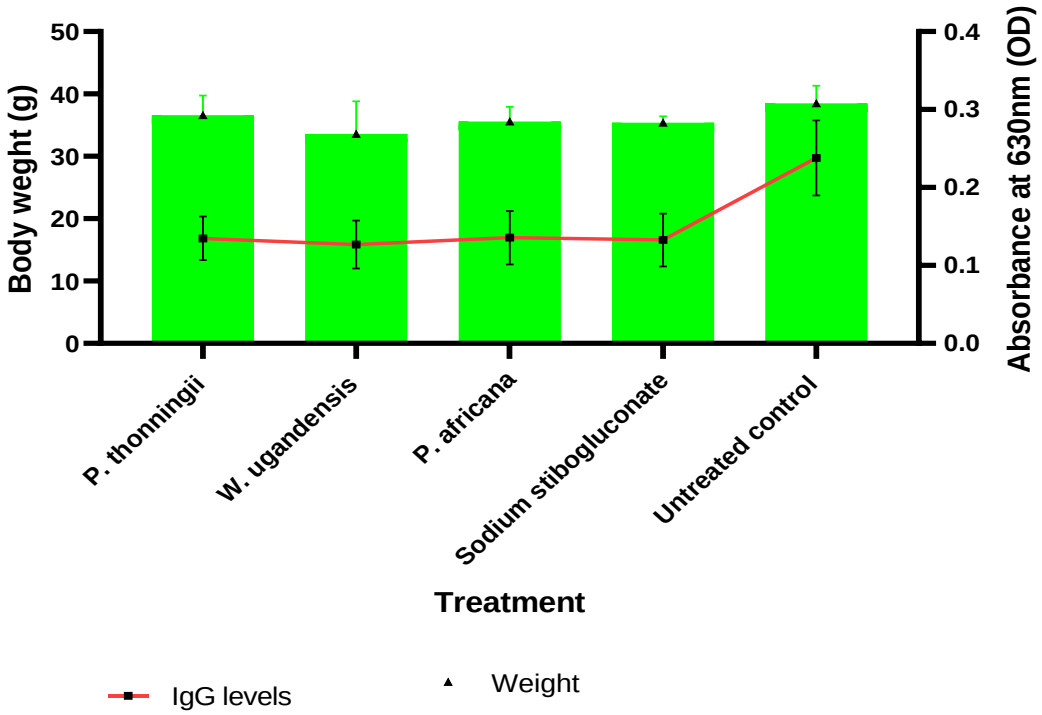

Figure 4.5: Relationship between body weights and IgG levels. Mean mice weights were taken on days 6, 12, and 18 during the treatment period. Body weight changes were determined by obtaining the difference of mice weights taken at day 0 and day 18, which was the last treatment day. Blood samples were taken from all mice for serum preparation for measurement of antileishmanial antibodies. The graph shows the relationship between mean mice weight and IgG production.

4.3 Parasite burden and its relationship with IgG antibodies

4.3.1 Parasite burden in splenic impressions

All mice were sacrificed a day after the last treatment day, and splenic impression smears were made. When viewed under a microscope, the splenic impression smears showed amastigotes within and outside of the splenocytes. The group treated with *W. ugandensis* had the lowest number of amastigotes at 13%, followed by sodium stibogluconate group with an infection rate of 14%. The percentage parasite counts in the mice treated with *P. thonningii* and *P. africana* were 23.2% and 30.4%, respectively (Figure 4.6). The outcome of treatments with *P. thonningii*, *W. ugandensis*, *Prunus africana*, and sodium stibogluconate were comparable ($P \geq 0.05$). All mice treated with both plant extracts and the untreated control drug showed significant differences ($P \leq 0.05$).

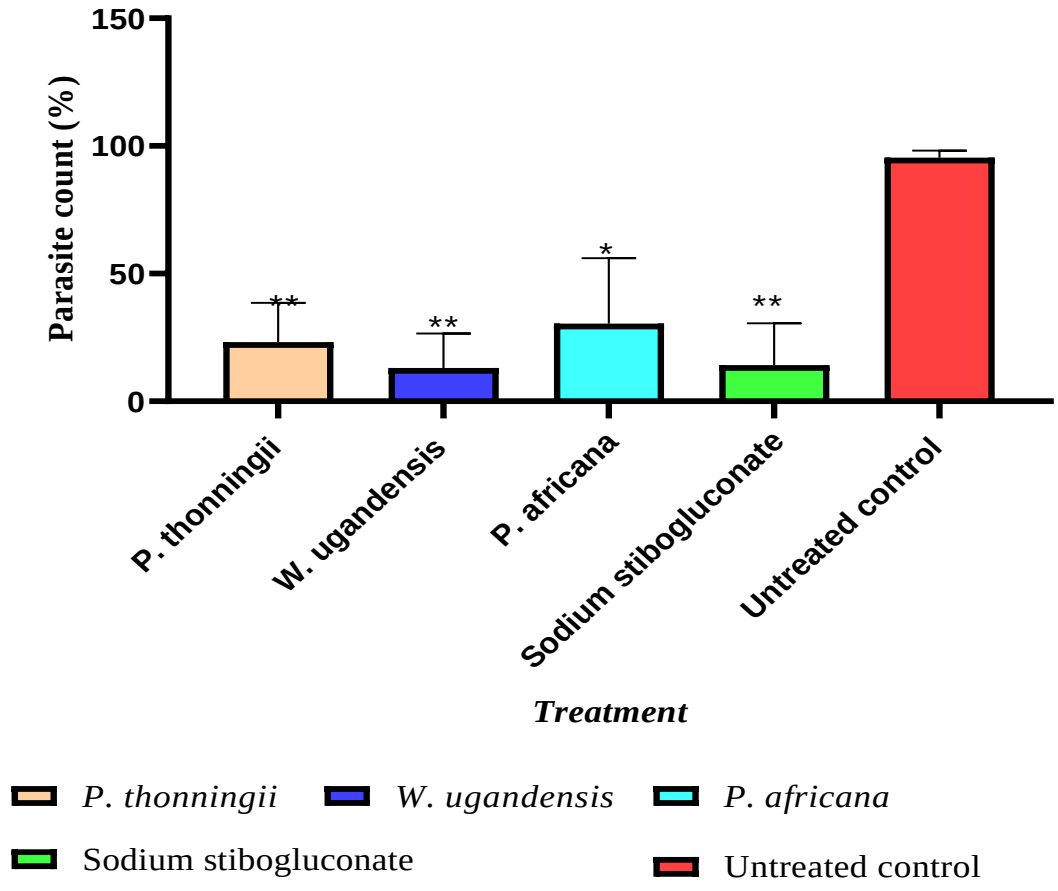

Figure 4.6: Percentage parasite count among the different treatment groups. The mice in each group were infected with *L. donovani* promastigotes and maintained for five weeks for disease development before commencement of treatment with the various plant extracts and control drugs. All mice were sacrificed a day after the last treatment, and splenic impression smears made. The number of amastigotes were obtained and expressed as percentage parasite count.

4.3.2 Relationship between parasite burden and levels of IgG antibodies

Following *L. donovani* infection and treatment of mice with various plant extracts and a control drug, the results showed a positive correlation between parasite load and the level of IgG produced. A higher percentage of parasite count was associated with a

higher absorbance (OD value). The untreated group, with the highest mean OD (0.2379) levels had the highest percentage of parasite count (95.4%). Groups treated with *W. ugandensis* and sodium stibogluconate had the lowest percentage parasite count and IgG production. There was a significant positive correlation between the mean percentage parasite count and IgG antibody production (r=0.9880, P=0.0016) (Figure 4.7).

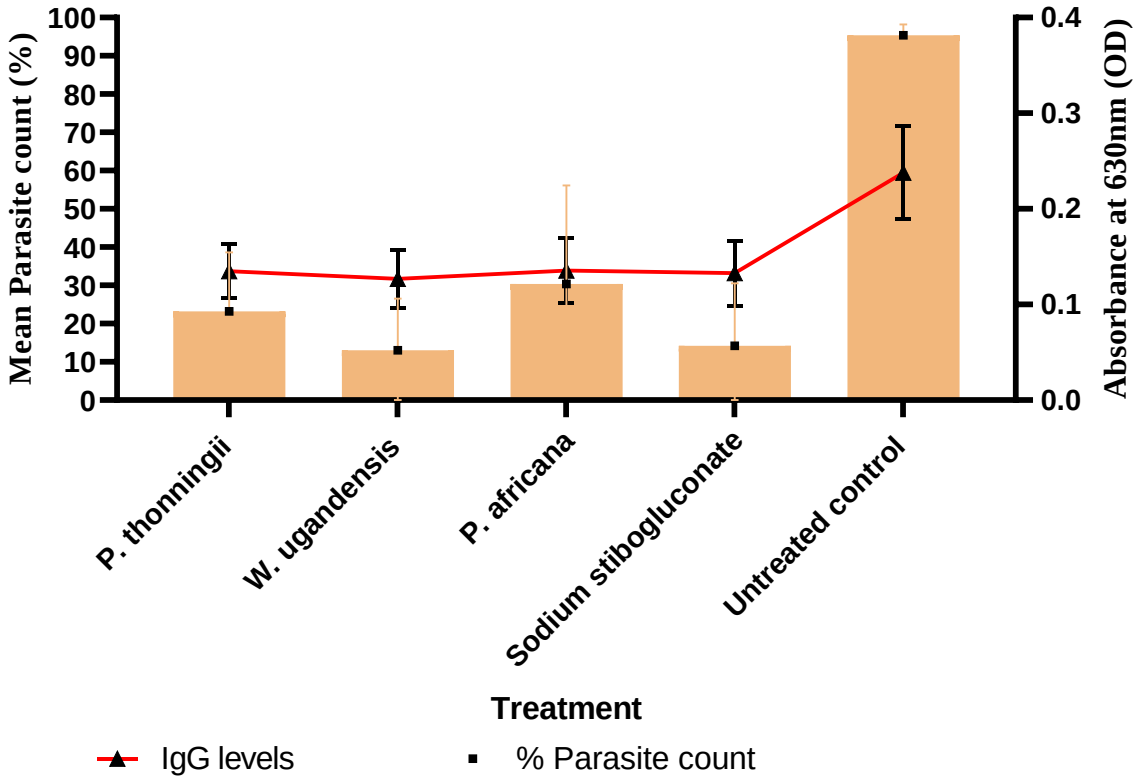

Figure 4.7: Relationship between parasite numbers and IgG levels. Mice were infected with *L. donovani* and left for five weeks for the disease to develop. They were treated with various plant extracts and a day after the last treatment, blood samples were taken from all mice for serum preparation for measurement of antileishmanial antibodies**.** Spleen was obtained for preparation of splenic impression smears for parasite quantification. The graph shows the relationship between parasite load and IgG production.

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

5.1.1 Antileishmanial activity of *Warburgia ugandensis, Prunus africana,* and *Piliostigma thonningii* extracts

Based on the results, *Warburgia ugandensis*, *Prunus africana*, and *Piliostigma thonningii* are promising compounds that can be used as alternative therapies for leishmaniasis. Besides, they were as good as the standard drug, sodium stibogluconate in the current study's findings. These findings support study findings reported by Githinji *et al*. (2010), who indicated that the bark of *Warburgia ugandensis* had significant antileishmanial activity against cutaneous leishmaniasis. *Warburgia ugandensis* also contains an alkaloid called muzigadial, which has trypanocidal activities against *Trypanosoma brucei*. Trypanosome and *Leishmania* parasites are kinetoplastids with similar structural and biochemical features and are therefore, likely to have similar responses to the same ethno-botanicals (Sakhuja and Kohli, 2018). Phytochemical studies conducted on *Piliostigma thonningii* reveal that it has various chemical compounds including flavonoids, tannins, alkaloids, volatile oils, and saponins, which confers it the vast activities as a medicinal plant (Ngure *et al*., 2014). Antileishmanial activity of *Prunus africana* has not been evaluated before; however, it has been shown to have antibacterial, antifungal, antimalarial, and anticancer activities (Githinji *et al*., 2010; Komakech and Kang, 2019; Deresa *et al*., 2022).

5.1.2 Cytotoxicity of *Warburgia ugandensis*, *Prunus africana*, and *Piliostigma thonningii* plant extracts

Pentostam and other current drugs for leishmaniasis are associated with high toxicity levels, which may have health implications. Often, most people avoid chemotherapy regimens for leishmaniasis due to the associated side effects. *Prunus africana*, *Warburgia ugandensis*, and *Piliostigma thonningii* are safe to use since they have less impact on Vero cells even in high concentrations. Other studies found the extracts of *Piliostigma thonningii* to be safe when administered orally (Olela *et al*., 2020). *Warburgia ugandensis* and *Prunus africana* have been proven to be safe even at a concentration that is higher than 250 µg/ml (Karani *et al*., 2013); hence, the natural products can be developed further as safe antileishmanial treatment.

5.1.3 Weight changes following *L donovani* infection and treatment of mice

Loss of weight is one of the many clinical symptoms of leishmaniasis and is attributed to loss of appetite and disturbed gastrointestinal function, which eventually leads to the subject being underweight (Feleke, 2019). Following a successful treatment regimen, patients are required to exhibit symptomatic improvement, including weight gain. The fact that all mice treated with the plant extracts and standard drug increased weight towards the last days of treatment indicates successful treatment and symptomatic relief. Though the increase in weight as treatment progressed may be attributed to drug activity in relieving symptoms, weight may not be used singly as a clinical parameter to test drug efficacy (Djimde *et al*., 2019). Besides, weight gain can also be attributed to

natural growth and nutrition, since both groups, including the naive control exhibited weight gain. However, the weight loss observed in the group treated with sodium stibogluconate between day zero and twelve may be linked to the drug toxicological effects; one of the side effects of sodium stibogluconate is loss of appetite and therefore, there are chances that the mice in this group were not feeding well, hence the weight loss.

5.1.4 Antileishmanial IgG production following treatment with the extracts of selected plants and its relationship with weight and parasite load

Though the production of IgG antibodies in the treatment groups was little, their presence indicates disease progression. The role of antibodies in leishmaniasis is not clear and there have been contradicting conclusions made by various researchers. According to Magalhães *et al*. (2021), high levels of anti-leishmanial IgG are indicative of active disease and correlate to high parasite burden. Other studies show that IgG antibodies do not protect against leishmaniasis but rather contribute to disease progression (De Lima *et al*., 2021). This might explain the high levels of IgG recorded in the untreated control. In the present study, the low levels of anti-leishmanial IgG antibodies recorded in the treated mice groups as compared to the untreated infected group may be an indication of the level of efficacies of the test products, which significantly reduced the parasite numbers. According to Miles *et al*. (2005), humoral responses during leishmaniasis infections do not play a major role in parasite clearance because the parasite hides in the lysosomes of the host, and hence protected from the host's immune system. However, the humoral immune responses are significant in

serological diagnosis of leishmaniasis. Therefore, a higher parasite load will trigger production of large amounts of IgG. The significant correlation between IgG antibodies and parasite numbers indicate that IgG levels may offer a possibility of the potential use of antibody levels in determining disease severity. *Leishmania donovani* parasites invade the spleen and liver, causing hepatomegaly and splenomegaly, which leads to increment of weight. The high mean weight of mice in the untreated control might be attributed to splenomegaly and hepatomegaly, associated with high parasitemia. However, the lack of significant correlation between antibody levels and body weight changes may indicate that antibody levels may not be used to predict body weight changes.

5.2 Conclusions

i. Methanolic extracts of the bark of *Warburgia ugandensis*, *Prunus africana,* and *Piliostigma thonningii* decreased the number of *L. donovani* promastigotes in culture.

ii. Methanolic extracts of the bark of *Prunus africana*, *Piliostigma thonningii,* and *Warburgia ugandensis* showed low levels of toxicity against Vero cells compared to the standard drug, sodium stibogluconate.

iii. Methanolic extracts of the bark of *Warburgia ugandensis*, *Prunus africana,* and *Piliostigma thonningii* decreased the number of *L. donovani* amastigotes in visceral leishmaniasis infected BALB/c

iv. Methanolic extracts of the bark of *Warburgia ugandensis*, *Prunus africana*, and *Piliostigma thonningii* have no significant effect on the weight of *Leishmania donovani* infected BALB/c mice.

v. Treatment with methanolic extracts of the bark of *Warburgia ugandensis*, *Prunus africana*, and *Piliostigma thonningii* decreased the amount of IgG production in *Leishmania donovani* infected BALB/c mice.

5.3 Recommendations

i. Since *Warburgia ugandensis*, *Prunus africana*, and *Piliostigma thonningii* showed high antileishmanial activities with less toxicological effects on host cells, researchers and pharmaceutical industries should develop oral formulations from these natural products to treat leishmaniasis in humans.

ii. *Warburgia ugandensis*, *Prunus africana*, and *Piliostigma thonningii* are promising alternative therapies against leishmaniasis. Therefore, local communities should grow more of the three trees to prevent their extinction.

5.4 Suggestions for further studies

i. Further research should be carried out in non-human primates to extrapolate the results obtained from mice studies.

ii. Further studies should be conducted to determine the plant metabolites that elicit anti-leishmanial activities in *Warburgia ugandensis*, *Prunus africana*, and *Piliostigma thonningii.*

iii. Future studies should be conducted using purified forms of *Warburgia ugandensis*, *Prunus africana*, and *Piliostigma thonningii* against *L. donovani* and other *Leishmania* forms.

REFERENCES

Abadías-Granado, I., Diago, A., Cerro, P., Palma-Ruiz, A., and Gilaberte, Y. (2021). Cutaneous and Mucocutaneous leishmaniasis. *Actas Dermo-Sifiliográficas (English Edition)*. https://doi.org/10.1016/j.adengl.2021.05.011.

Abreu-Silva, L. (2013). In vitro antileishmanial activity of essential oil of *Vanillosmopsis arborea* (Asteraceae) baker. *Evidence based Complementary Alternative Medicine,* 2013:727

Afolayan, M., Srivedavyasasri, R., Asekun, O. T., Familoni, O. B., Orishadipe, A., Zulfiqar, F., Ibrahim, M. A., and Ross, S. A. (2018). Phytochemical study of *Piliostigma thonningii*, a medicinal plant grown in Nigeria. *Medicinal Chemistry Research, 27*(10), 2325-2330. https://doi.org/10.1007/s00044-018-2238-1

Alemayehu, B., and Alemayehu, M. (2017). Leishmaniasis: A review on parasite, vector, and reservoir host. *Health Science Journal*, *11*(4). https://doi.org/10.21767/1791-809x.1000519

Ali Al- Hajj, M. M., Al-Shamahy, H. A., Alkhatib, B. Y., and Moharram, B. A. (2018). In vitro anti-leishmanial activity against cutaneous leishmania parasites and preliminary phytochemical analysis of four Yemeni medicinal plants. *Universal Journal of Pharmaceutical Research*. https://doi.org/10.22270/ujpr.v3i4.183

Alviano, D. S., Barreto, A. L., Dias, F. D., Rodrigues, I. D., Rosa, M. D., Alviano, C. S., and Soares, R. M. (2012). Conventional therapy and promising plant-derived compounds against Trypanosomatid parasites. *Frontiers in Microbiology*, *3*. https://doi.org/10.3389/fmicb.2012.00283.

Arenas, R., and Juarez-Duran, E. R. (2020). Faculty opinions recommendation of *Leishmania* vaccines entered in clinical trials: A review of literature. *Faculty Opinions – Post-Publication Peer Review of Biomedical Literature*. https://doi.org/10.3410/f.739007264.793580959.

Badirzadeh, A., Heidari-Kharaji, M., Fallah-Omrani, V., Dabiri, H., Araghi, A., and Salimi Chirani, A. (2020). Antileishmanial activity of *Urtica dioica* extract against zoonotic cutaneous leishmaniasis. *PLOS Neglected Tropical Diseases*, *14*(1), e0007843. https://doi.org/10.1371/journal.pntd.0007843.

Bezerra, J. M., De Araújo, V. E., Barbosa, D. S., Martins-Melo, F. R., Werneck, G. L., and Carneiro, M. (2018). Burden of leishmaniasis in Brazil and federated units, 1990-2016: Findings from global burden of disease study 2016. *PLOS Neglected Tropical Diseases*, *12*(9), e0006697. https://doi.org/10.1371/journal.pntd.0006697.

Bi, K., Chen, Y., Zhao, S., Kuang, Y., and John Wu, C. (2018). Current visceral leishmaniasis research: A research review to inspire future study. *BioMed Research International*, *2018*, 1-13. https://doi.org/10.1155/2018/9872095

Brito, R., Guimarães, F., Velloso, J., Corrêa-Oliveira, R., Ruiz, J., Reis, A., and Resende, D. (2017). Immunoinformatic features linked to leishmania vaccine development: Data integration of experimental and in Silico studies. *International Journal of Molecular Sciences*, *18*(2), 371. https://doi.org/10.3390/ijms18020371.

Burza, S., Croft, S. L., and Boelaert, M. (2018). Leishmaniasis. *The Lancet*, *392*(10151), 951-970. https://doi.org/10.1016/s0140-6736(18)31204-2

Cecílio, P., Cordeiro-da-Silva, A., and Oliveira, F. (2022). Sand flies: Basic information on the vectors of leishmaniasis and their interactions with leishmaniaparasites. *Communications Biology*, *5*(1). https://doi.org/10.1038/s42003-022-03240-z

Chakravarty, J., and Sundar, S. (2019). Current and emerging medications for the treatment of leishmaniasis. *Expert Opinion on Pharmacotherapy*, *20*(10), 1251-1265. https://doi.org/10.1080/14656566.2019.1609940.

Chappuis, F., Sundar, S., Hailu, A., Ghalib, H., Rijal, S., Peeling, R. W., Alvar, J., and Boelaert, M. (2007). Visceral leishmaniasis: What are the needs for diagnosis, treatment, and control? *Nature Reviews Microbiology*, *5*(11), 873-882. https://doi.org/10.1038/nrmicro1748.

Cobb, L. (2019). Cell proliferation assays and cell viability assays. *Materials and Methods*, *9*. https://doi.org/10.13070/mm.en.9.2799.

Colares, V., Almeda-Souza, F., Taniwaki, N., Souza Cda, S., da Costa, G., Calabrese Kda, S and Ngure, K., Kimutai, A., Ng'ang'a, W., Rukunga, G., and Tonui, K. (2009). A review of Leishmaniasis in Eastern Africa. *Journal of Biomedical Research* 29(3): 214-223.

De Lima, C. M., Magalhães, A. S., Costa, R., Barreto, C. C., Machado, P. R., Carvalho, E. M., Lessa, M. M., and Carvalho, L. P. (2021). High anti-leishmania IgG antibody levels are associated with severity of mucosal leishmaniasis. *Frontiers in Cellular and Infection Microbiology*, *11*. https://doi.org/10.3389/fcimb.2021.652956.

Deresa, D. A., Abdissa, Z., Gurmessa, G. T., and Abdissa, N. (2022). Chemical constituents of the stem bark of *Prunus africana* and evaluation of their antibacterial activity. *Journal of the Turkish Chemical Society Section A: Chemistry*, *9*(2), 395-414. https://doi.org/10.18596/jotcsa.1029564.

Djimde, M., Samouda, H., Jacobs, J., Niangaly, H., Tekete, M., Sombie, S. B., Mgina, E. J., Fofana, B., Sagara, I., Doumbo, O. K., Vaillant, M., and Djimde, A. A. (2019). Relationship between weight status and anti-malarial drug efficacy and safety in children in Mali. *Malaria Journal*, *18*(1). https://doi.org/10.1186/s12936-019-2673-6.

Feleke, B. E. (2019). Nutritional status of visceral leishmaniasis patients: A comparative cross-sectional study. *Clinical Nutrition ESPEN*, *33*, 139-142. https://doi.org/10.1016/j.clnesp.2019.06.005.

Gamboa-Leon, R., Vera-Ku, M., Peraza-Sanchez, S. R., Ku-Chulim, C., Horta-Baas, A., and Rosado-Vallado, M. (2014). Antileishmanial activity of a mixture of *Tridax procumbens* and (Allium sativum) in mice. *Parasite*, *21*, 15. https://doi.org/10.1051/parasite/2014016.

Gidey, K. (2020). Visceral leishmaniasis treatment outcome and associated factors in northern Ethiopia. *International Journal of Infectious Diseases*, *101*, 420-421. https://doi.org/10.1016/j.ijid.2020.09.1103.

Gitari, J. W., Nzou, S. M., Wamunyokoli, F., Kinyeru, E., Fujii, Y., Kaneko, S., and Mwau, M. (2018). *Leishmaniasis recidivan*s by *Leishmania tropica* in central Rift Valley region in Kenya. *International Journal of Infectious Diseases*, *74*, 109-116. https://doi.org/10.1016/j.ijid.2018.07.008.

Githinji, E., Irungu, L., Tonui, W., Rukunga, G., Mutai, C., Muthaura, C., Lugalia, R., Gikandi, G., Wainaina, C., Ingonga, J., and Wanjoya, A. (2010). In vitro effects of *Warburgia ugandensis, Psiadia punctulata* and *Chasmanthera dependens* on *Leishmania major* promastigotes. *African Journal of Traditional, Complementary and Alternative Medicines*, *7*(3). https://doi.org/10.4314/ajtcam.v7i3.54791.

Glans, H., Dotevall, L., Söbirk, S. K., Färnert, A., and Bradley, M. (2018). Cutaneous, mucocutaneous, and visceral leishmaniasis in Sweden from 1996–2016: A retrospective study of clinical characteristics, treatments, and outcomes. *BMC Infectious Diseases*, *18*(1). https://doi.org/10.1186/s12879-018-3539-1.

Goto, H. (2012). Review of the current treatments for leishmaniases. *Research and Reports in Tropical Medicine*, 69. https://doi.org/10.2147/rrtm.s24764

Hailu, A., Dagne, D. A., and Boelaert, M. (2016). Leishmaniasis. *Neglected Tropical Diseases - Sub-Saharan Africa*, 87-112. https://doi.org/10.1007/978-3-319-25471-5_5.

Ibrahim, H., Hamid, K. A., Aziz, T. A., Samir El bahwashy, A., Khattab, H., Aaref, B., and Elsayed, E. (2023). Nasal mucocutaneous leishmaniasis (MCL) with necrotizing granulomatous inflammation inducing cytotoxic T-cell lymphoma in a male yemeni patient. *The Egyptian Journal of Internal Medicine*, *35*(1). https://doi.org/10.1186/s43162-023-00219-y.

Ickenstein, L. M., and Garidel, P. (2019). Lipid-based nanoparticle formulations for small molecules and RNA drugs. *Expert Opinion on Drug Delivery*, *16*(11), 1205-1226. https://doi.org/10.1080/17425247.2019.1669558.

An, I., Harman, M., Esen, M., and Çelik, H. (2019). The effect of pentavalent antimonial compounds used in the treatment of cutaneous leishmaniasis on hemogram and biochemical parameters. *Cutaneous and Ocular Toxicology*, *38*(3), 294-297. https://doi.org/10.1080/15569527.2019.1610887.

Jumba, B. N., Anjili, C. O., Makwali, J., Ingonga, J., Nyamao, R., Marango, S., Choge, J. K., and Khayeka-Wandabwa, C. (2015). Evaluation of leishmanicidal activity and cytotoxicity of *Ricinus communis* and *Azadirachta indica* extracts from western Kenya: In vitro and in vivo assays. *BMC Research Notes*, *8*(1). https://doi.org/10.1186/s13104-015-1605-y.

Karani, L., Tolo, F., Karanja, S., and Khayeka−Wandabwa, C. (2013). Safety of *Prunus africana* and *Warburgia ugandensis* in asthma treatment. *South African Journal of Botany*, *88*, 183-190. https://doi.org/10.1016/j.sajb.2013.07.007.

Kayani, B., Sadiq, S., Rashid, H. B., Ahmed, N., Mahmood, A., Khaliq, M. S., Maqsood, R., Rashid, H., Hasan, S., Mushtaq, M. H., Zia, U., and Chaudhry, M. (2021). Cutaneous leishmaniasis in Pakistan: A neglected disease needing one health strategy. *BMC Infectious Diseases*, *21*(1). https://doi.org/10.1186/s12879-021-06327-w.

Kishore, M. (2017). Visceral leishmaniasis in an Immunocompromised patient: Diagnostic bone marrow Cytomorphology. *Journal of Clinical and Diagnostic Research*. https://doi.org/10.7860/jcdr/2017/28061.10013.

Kokwaro, J. (2009). Medicinal plants of East Africa. East African Publishing Bureau, Nairobi. 3rd Edition.

Komakech, R., and Kang, Y. (2019). Ethnopharmacological potential of African cherry (*Prunus africana*). *Journal of Herbal Medicine*, *17-18*, 100283. https://doi.org/10.1016/j.hermed.2019.100283

Kone, W., Azokou, A., Bakayoko, A., and Bi, F. T. (2012). Preliminary study on polysaccharides and certain secondary metabolites of medicinal plants used in Cote D'Ivoire for wound healing. *Research Journal of Medicinal Plant*, *6*(3), 214-224. https://doi.org/10.3923/rjmp.2012.214.224.

Kumar, A. (2021). Immunological determinants, host immune responses, and animal models of visceral leishmaniasis. *Pathogenesis, Treatment and Prevention of Leishmaniasis*, 231-250. https://doi.org/10.1016/b978-0-12-822800-5.00012-3.

Lockwood, D., and Moore, E. (2010). Treatment of visceral leishmaniasis. *Journal of Global Infectious Diseases*, *2*(2), 151. https://doi.org/10.4103/0974-777x.62883.

Magalhães, A., Carvalho, L. P., Costa, R., Pita, M. S., Cardoso, T. M., Machado, P. R., Carvalho, E. M., Arruda, S., and Carvalho, A. M. (2021). Anti-leishmania IgG is a marker of disseminated leishmaniasis caused by *Leishmania braziliensis*. *International Journal of Infectious Diseases*, *106*, 83-90. https://doi.org/10.1016/j.ijid.2021.02.016.

Malvolti, S., Malhame, M., Mantel, C., Rutte, E. A., and M.Kaye, P. (2021). Human leishmaniasis vaccines: Use cases, target population and potential global demand. https://doi.org/10.1101/2021.07.09.21260268.

Mann, S., Frasca, K., Scherrer, S., Henao-Martínez, A. F., Newman, S., Ramanan, P., and Suarez, J. A. (2021). A review of leishmaniasis: Current knowledge and future directions. *Current Tropical Medicine Reports*, *8*(2), 121-132. https://doi.org/10.1007/s40475-021-00232-7.

Marango, S. N., Khayeka-Wandabwa, C., Makwali, J. A., Jumba, B. N., Choge, J. K., Adino, E. O., and Anjili, C. O. (2017). Experimental therapeutic assays of *Tephrosia vogelii* against *Leishmania major* infection in murine model: In vitro and in vivo. *BMC Research Notes*, *10*(1). https://doi.org/10.1186/s13104-017-3022-x.

Mazire, P., Agarwal, V., and Roy, A. (2021). Roadmap of pre-clinical treatment for visceral leishmaniasis. *Drug Development Research*, *83*(2), 317-327. https://doi.org/10.1002/ddr.21907.

McGwire, B. S., and Satoskar, A. R. (2013). Leishmaniasis: Clinical syndromes and treatment. *QJM*, *107*(1), 7-14. https://doi.org/10.1093/qjmed/hct116.

Mears, E. R., Modabber, F., Don, R., and Johnson, G. E. (2015). A review: The current in vivo models for the discovery and utility of new anti-leishmanial drugs targeting cutaneous leishmaniasis. *PLOS Neglected Tropical Diseases*, *9*(9), e0003889. https://doi.org/10.1371/journal.pntd.0003889

Mehrolhasani, N. (2014). Diffuse cutaneous leishmaniasis in HIV positive woman. *Our Dermatology Online*, *5*(2), 197-197. https://doi.org/10.7241/ourd.20142.49.

Miles, S. A., Conrad, S. M., Alves, R. G., Jeronimo, S. M., and Mosser, D. M. (2005). A role for IgG immune complexes during infection with the intracellular pathogen *Leishmania. The Journal of Experimental Medicine, 201*(5), 747-754. https://doi.org/10.1084/jem.20041470.

Monge-Maillo, B., and Lopez-Velez, R. (2015). Miltefosine for visceral and cutaneous leishmaniasis: Drug characteristics and evidence-based treatment recommendations. *Clinical Infectious Diseases*. https://doi.org/10.1093/cid/civ004.

Moraes Neto, R. N., Setúbal, R. F., Higino, T. M., Brelaz-de-Castro, M. C., Da Silva, L. C., and Aliança, A. S. (2019). Asteraceae plants as sources of compounds against leishmaniasis and Chagas disease. *Frontiers in Pharmacology*, *10*. https://doi.org/10.3389/fphar.2019.00477.

Mutiso, J. M., Macharia, J. C., Barasa, M., Taracha, E., Bourdichon, A. J., and Gicheru, M. M. (2011). In vitro and in vivo antileishmanial efficacy of a combination therapy of diminazene and artesunate against *Leishmania donovani* in BALB/C mice. *Revista do Instituto de Medicina Tropical de São Paulo*, *53*(3), 129-132. https://doi.org/10.1590/s0036-46652011000300003.

Ngure, P. K., Kimutai, A., Ng'ang'a, Z. W., Rukunga, G., and Tonui, W. K. (2009). A review of leishmaniasis in eastern Africa. *Journal of Nanjing Medical University*, *23*(2), 79-86. https://doi.org/10.1016/s1007-4376(09)60032-0.

Ngure, P., Ng'ang'a, Z., Kimutai, A., Kepha, S., Mong'are, S., Ingonga, J., and Tonui, W. (2014). Immunostimulatory responses to crude extracts of *Warburgia ugandensis* (Sprague) subsp *ugandensis* (*canellaceae*) by Balb/C mice infected with *Leishmania major*. *Pan African Medical Journal*, *17*. https://doi.org/10.11604/pamj.supp.2014.17.1.3638.

Olela, B., Mbaria, J., Wachira, T., and Moriasi, G. (2020). Acute oral toxicity and anti-inflammatory and analgesic effects of aqueous and Methanolic stem bark extracts of *Piliostigma thonningii* (Schumach.). *Evidence-Based Complementary and Alternative Medicine*, *2020*, 1-10. https://doi.org/10.1155/2020/5651390.

Oryan, A. (2015). Plant-derived compounds in treatment of leishmaniasis. *Iranian Journal of Veterinary Research*, 16(1), 1–19. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/27175144

Osada, Y., Omachi, S., Sanjoba, C., and Matsumoto, Y. (2016). Animal models of visceral leishmaniasis and applicability to disease control. *Kala Azar in South Asia*, 287-296. https://doi.org/10.1007/978-3-319-47101-3_23.

Ouma, F. F., and Mulambalah, C. S. (2021). Persistence and changing distribution of leishmaniases in Kenya require a paradigm shift. *Journal of Parasitology Research, 2021*, 1-6. https://doi.org/10.1155/2021/9989581.

Pace, D., Williams, T. N., Grochowska, A., Betts, A., Attard-Montalto, S., Boffa, M. J., and Vella, C. (2011). Manifestations of pediatric *Leishmania infantum* infections in Malta. *Travel Medicine and Infectious Disease*, *9*(1), 37-46. https://doi.org/10.1016/j.tmaid.2010.11.005.

Parasites without borders. (2020, **February 6).** Education for Parasitic Diseases. Parasites Without Borders. https://parasiteswithoutborders.com/the-problem/.

Ready, P. (2014). Epidemiology of visceral leishmaniasis. *Clinical Epidemiology*, 147. https://doi.org/10.2147/clep.s44267.

Reguera, R. M., Pérez-Pertejo, Y., Gutiérrez-Corbo, C., Domínguez-Asenjo, B., Ordóñez, C., García-Estrada, C., Martínez-Valladares, M., and Balaña-Fouce, R. (2019). Current and promising novel drug candidates against visceral leishmaniasis. *Pure and Applied Chemistry*, *91*(8), 1385-1404. https://doi.org/10.1515/pac-2018-1102.

Reimão, J. Q., Coser, E. M., Lee, M. R., and Coelho, A. C. (2020). Laboratory diagnosis of cutaneous and visceral leishmaniasis: Current and future methods. *Microorganisms*, *8*(11), 1632. https://doi.org/10.3390/microorganisms8111632.

Reimão, J. Q., Coser, E. M., Lee, M. R., and Coelho, A. C. (2020). Laboratory diagnosis of cutaneous and visceral leishmaniasis: Current and future methods. *Microorganisms*, *8*(11), 1632. https://doi.org/10.3390/microorganisms8111632.

Rossi, M., and Fasel, N. (2017). How to master the host immune system? Leishmania parasites have the solutions! *International Immunology*, *30*(3), 103-111. https://doi.org/10.1093/intimm/dxx075.

Sachdeva, H., Sehgal, R., and Kaur, S. (2014). *Tinospora cordifolia* as a protective and immunomodulatory agent in combination with cisplatin against murine visceral leishmaniasis. *Experimental Parasitology*, *137*, 53-65. https://doi.org/10.1016/j.exppara.2013.12.006

Sakhuja, V., and Kohli, H. S. (2018). Leishmaniasis and trypanosomiasis. *Oxford Medicine Online*. https://doi.org/10.1093/med/9780199592548.003.0184_update_001.

Savoia, D. (2015). Recent updates and perspectives on leishmaniasis. *The Journal of Infection in Developing Countries*, *9*(06), 588-596. https://doi.org/10.3855/jidc.6833.

Selvapandiyan, A., Croft, S. L., Rijal, S., Nakhasi, H. L., and Ganguly, N. K. (2019). Innovations for the elimination and control of visceral leishmaniasis. *PLOS Neglected Tropical Diseases*, *13*(9), e0007616. https://doi.org/10.1371/journal.pntd.0007616.

Singh, O. P., and Sundar, S. (2015). Developments in diagnosis of visceral leishmaniasis in the elimination era. *Journal of Parasitology Research*, *2015*, 1-10. https://doi.org/10.1155/2015/239469.

Srivastava, P., Dayama, A., Mehrotra, S., and Sundar, S. (2011). Diagnosis of visceral leishmaniasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *105*(1), 1-6. https://doi.org/10.1016/j.trstmh.2010.09.006.

Sundar, S. (2016). Treatment of visceral leishmaniasis. *International Journal of Infectious Diseases*, *45*, 54. https://doi.org/10.1016/j.ijid.2016.02.164.

Sundar, S., and Agarwal, D. (2018). Visceral leishmaniasis—Optimum treatment options in children. *Pediatric Infectious Disease Journal*, *37*(5), 492-494. https://doi.org/10.1097/inf.0000000000001885.

Sundar, S., and Singh, O. P. (2018). Molecular diagnosis of visceral leishmaniasis. *Molecular Diagnosis & Therapy*, *22*(4), 443-457. https://doi.org/10.1007/s40291-018-0343-y.

Sunyoto, T., Adam, G. K., Atia, A. M., Hamid, Y., Babiker, R. A., Abdelrahman, N., Vander Kelen, C., Ritmeijer, K., Alcoba, G., Den Boer, M., Picado, A., and Boelaert, M. (2018). "Kala-Azar is a dishonest disease": Community perspectives on access barriers to visceral leishmaniasis (Kala-Azar) diagnosis and care in southern Gadarif, Sudan. *The American Journal of Tropical Medicine and Hygiene*, *98*(4), 1091-1101. https://doi.org/10.4269/ajtmh.17-0872.

Thakur, S., Joshi, J., and Kaur, S. (2020). Leishmaniasis diagnosis: An update on the use of parasitological, immunological, and molecular methods. *Journal of Parasitic Diseases*, *44*(2), 253-272. https://doi.org/10.1007/s12639-020-01212-w

Torres-Guerrero, E., Quintanilla-Cedillo, M. R., Ruiz-Esmenjaud, J., and Arenas, R. (2017). Leishmaniasis: A review. *F1000Research*, *6*, 750. https://doi.org/10.12688/f1000research.11120.1.

Trindade, M. A., Silva, L. L., Braz, L. M., Amato, V. S., Naafs, B., and Sotto, M. N. (2015). Post-kala-azar dermal leishmaniasis and leprosy: Case report and literature review. *BMC Infectious Diseases*, *15*(1). https://doi.org/10.1186/s12879-015-1260-x.

Wiwanitkit, V. (2012). Interest in paromomycin for the treatment of visceral leishmaniasis (kala-Azar). *Therapeutics and Clinical Risk Management*, 323. https://doi.org/10.2147/tcrm.s30139.

World health organization (WHO). (2022). Leishmaniasis. Available at: https://www.who.int/news-room/fact-sheets/detail/leishmaniasis.

APPENDICES

Appendix I: Images of *Warburgia Ugandensis*

Appendix II: Images of *Piliostigma thonningii*

Appendix III: Images of *Prunus africana*

Appendix IV: Ethical Approval

KENYATTA UNIVERSITY
ETHICS REVIEW COMMITTEE

Fax: 8711242 8711575
Email: kuerc.chairman@ku.ac.ke
kuerc.secretary@ku.ac.ke
Website: www.ku.ac.ke

P. O. Box 43844,
Nairobi, 00100
Tel: 8710901/12

Our Ref: KU/ERC/ COND. APPROVAL/VOL.1 Date: 16th August. 2018

PROF. MIACHAEL GICHERU
P.O Box 43844, 00100
Nairobi.

Dear Gicheru,

APPLICATION NUMBER: PKU/848/1913 "EPIDEMIOLOGY OF LEISHMANIASIS IN WEST POKOT AND DEVELOPMENT OF STRATEGIES FOR EFFECTIVE DISEASE CONTROL"

1. IDENTIFICATION OF PROTOCOL

The application before the committee is with a research topic **"Epidemiology of Leishmaniasis in West Pokot and Development of Strategies for Effective Disease Control"** received on 12th June, 2018 and discussed on 14th August, 2018

2. APPLICANT

Prof. Miachael Gicheru

3. SITE

West Pokot

4. DECISION

The committee has considered the research protocol in accordance with the Kenyatta University Research Policy (section 7.2.1.3) and the Kenyatta University Ethics Review Committee Guidelines and **APPROVED that the research may proceed ON CONDITION that you incorporate its advice as below.**

5. ADVICE/CONDITIONS